Dynamic Regulation of Bacterial Metabolic Pathways using Autonomous, Pathway-Independent Control Strategies

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Abstract

Metabolic engineering efforts have so far focused on strain optimization through careful metabolic modeling and tinkering with host genomes, through gene knockouts or knockins, to direct flux in desired channels. These efforts have borne fruit with the development of large manufacturing processes for numerous chemicals. The next challenge for metabolic engineering, however, lies in tackling issues associated with construction of more complex pathways, such as those that directly interfere with host metabolism, have branchpoints with promiscuous enzymes, or synthesize toxic intermediates or products. Dynamic metabolic engineering has emerged as a new frontier for tool development to allow regulation and control of native and cellular pathways during the course of a production run. Advantages in dynamic strategies are especially apparent in the aforementioned examples where traditional static strategies of gene knockouts or knockins are not an option. Instead, it is necessary to be able to control when certain genes are expressed, such as to build biomass before switching on growth-limiting production pathways, or accumulating intermediates to drive the reaction of a promiscuous enzyme along a certain branch.

In this thesis, we propose enzyme control strategies that are independent of any biosynthetic pathway of interest. Therefore, they can theoretically be applied to a wide variety of contexts in a "plug-and-play" fashion to control pathway enzyme expression. After initial work to understand the limitations of nutrient starvation strategies to induce genetic circuits, we decided to use quorum sensing circuitry to create circuits that can be autonomously induced. We used parts of the Esa QS system (derived from Pantoea stewartii) to create circuit variants in the Escherichia coli genome, which switch off expression of the targeted gene at various times and cell densities. Switching times were varied by modulating the expression of the AHL synthase, and therefore the production rate of AHL, the quorum sensing molecule. Switching dynamics were characterized and ranked for the entire library of circuit variants using fluorescent reporters. The characterized device was used to identify optimal switching times for redirection of glycolytic fluxes into heterologous pathways, resulting in a 5.5-fold boost in myo-inositol (MI) and increasing glucaric acid titer from unmeasurable quantities up to >0.8 g/L. With a focus on industrial application, consistency of device performance was verified in benchtop bioreactors, achieving nearly 10-fold and 5-fold boosts in specific titers of myo-inositol and glucaric acid, respectively. To demonstrate broad utility and "off-the-shelf" applicability, the control module was applied to dynamic downregulation of flux into aromatic amino acid biosynthesis to accumulate the industrially-relevant intermediate, shikimate, resulting in an increase in titers from unmeasurable quantities to >100 mg/L. Finally, this QS device was coupled with a MI-biosensor circuit to institute two layers of dynamic regulation and further improve glucaric acid titers. Production trials in these composite strains resulted in the highest glucaric acid titers (~2 g/L) reported to date from E. coli K-strains.

This work reports the first completely autonomous dynamic regulation module and its application in bioproduction of multiple products from different metabolic pathways. We envision that the strategy presented here may be adapted to any pathway context and gene of interest. With increased prevalence of dynamic regulation, the relevant strategies may become standardized for general use.

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Chapter 1

Introduction
1.1 Microbial Chemical Factories

Production of useful compounds from biological hosts has been explored extensively in the last several decades. The field of biotechnology has advanced from production of simpler compounds, such as lactic acid and ethanol, to more complex products, such as therapeutic antibodies. While a range of hosts have been explored, microorganisms still remain as the predominant fraction as opposed to their mammalian or plant counterparts. Reasons include the ease of genetic manipulation, short life times, ease of culture and potentially higher titers. In addition, newer feedstocks are being explored for consumption by microbes, such as lignocellulose, which could potentially further reduce the cost of microbial bioproduction processes.

Typical developmental cycles for microbial production processes include pathway development and library testing, host/strain selection and optimization, media development and optimization, and scale-up to maximize productivity. Downstream processing includes extraction, purification and concentration of the product, as needed. The advantage of using microbial hosts include environmentally friendly feedstocks and potentially lower capital and operational costs due to the lack of complex starting material, catalysts or extreme conditions. Many compounds, such as polyhydroxyalkanoates, are chiral and are therefore much more easily synthesized in microbial systems than chemical systems. Others, such as D-glucaric acid, can be made chemically, but only through a nonselective and expensive process using nitric acid as an oxidant. Thus, microbial routes are favorable in many cases and have thus been explored extensively in the last several decades for compounds of increasing complexity.

1.2 Traditional Metabolic Engineering

Metabolic engineering efforts so far have focused largely on directing flux along pathways that generate the optimal amount of product. Typically, this is aided through metabolic modeling such as Flux Balance Analysis (FBA), which finds the steady state flux distribution given the genetic profile of the host. The assumption here is that the system performs at steady state and does not undergo growth-dependent changes in metabolism. Steady state mass balances for each metabolite form a set of linear equations that can be easily solved, given the appropriate constraints. This may be especially valid in cases of continuous culture where growth rate is kept constant, however, the assumptions do break down in parts of batch and fed-batch processes when the concentration of the extracellular carbon source is necessarily unsteady. Nonetheless, FBA has been used extensively in conjunction with optimization algorithms (such as Optknock or OptFlux) to determine gene knockouts or bottlenecks that could be relieved to maximize product formation.
Host metabolic activity is heavily regulated through gene expression and its regulation. More complex models that include transcriptional regulation have thus been developed to more accurately capture system behavior and increase predictive power\textsuperscript{7,8}. In addition, dynamic FBA models relax the steady state assumption of traditional FBA to capture temporal effects from changing environments, such as decreasing nutrients, over the growth cycle\textsuperscript{9}.

In addition to model-guided host optimization, simple rational engineering has also borne tremendous success. Modular metabolic engineering has been a widely applied approach whereby a metabolic pathway is rationally divided into different modules. The expression of these modules can then be varied combinatorially or pseudo-rationally to find the optimal expression profile along the metabolic pathway that maximizes production\textsuperscript{10-12}. Typically, these modules are placed on different plasmids under different promoters. Plasmid copy numbers and promoter strengths can be varied to change module expression level. Thus, a combination of gene knockouts and the introduction of modular heterologous pathways have comprised most metabolic engineering strategies to date.

1.3 Interfacing Synthetic Biology Strategies

Recently, synthetic biology has emerged as a new frontier to provide complex control strategies for metabolic engineering. These are especially useful when bioproduction of a certain compound cannot be simply resolved through introduction of a heterologous pathway. Dunlop et al. provide an example of the challenge in producing 1-butanol, which is highly toxic to the host bacteria\textsuperscript{13}. They computationally illustrate how certain biological circuit design principles, such as introduction of feed-forward loops to rapidly produce butanol efflux pumps, butanol-responsive promoters, and repressor cascades can be utilized to reduce the toxicity that the host faces. This leads to higher predicted product titers. In addition, RNA-based gene control schemes have also been proposed as powerful multiplexed methods to control and tune expression of multiple genes in a metabolic pathway\textsuperscript{14}.

The true power of synthetic biology interfaced with metabolic engineering, however, lies in allowing the host to take cues from the environment, processing this information, and then adjusting its behavior to optimize the desired objective function, such as maximizing product formation. Thus, dynamically regulating system pathways, both native and heterologous, can allow continuous adjustment such that the system always remains at the point of optimal performance even in the face of environmental heterogeneities.

1.4 Dynamic Metabolic Engineering
1.4.1 Motivation for Dynamic Pathway Regulation

While static strategies, such as gene knockouts, expression level tuning, and protein evolution have been successful, they are unable to address challenges associated with more complex pathways and products. In these cases, static strategies to generate an optimal host are not an option, and it is necessary to regulate and change metabolic behavior during the course of a production run. Examples include cases where essential cell pathways reduce efficiency of product formation pathways by drawing from common metabolite pools, promiscuous enzymes operate on successive intermediates in a pathway, or the end-product is toxic to cells. Not surprisingly, the idea of "just-in-time transcription" is prevalent in natural systems also, such as amino acid biosynthesis, where temporal distribution of different enzymes along a metabolic pathway reduces metabolic burden. Promoter activity for enzymes in the beginning of amino acid biosynthesis pathways were shown to increase more rapidly in response to amino acid starvation than those of downstream enzymes.

In the case of heterologous pathways, quantitative justification for dynamic regulation has been amply provided by several computational models. Gadkar et al demonstrated a bilevel optimization strategy for the two cases of production of glycerol and ethanol. Glycerol production branches off from central carbon metabolism, and diverting flux towards its synthesis requires shutting off genes that contribute to growth precursors. In the case of ethanol production, acetate synthesis/ATP generation competes with ethanol production. Diverting acetate flux towards ethanol also compromises ATP generation, causing a direct inhibition of growth. The authors showed that in both these cases, it was advantageous to have a dynamic regulation strategy which shuts off essential genes mid-way through the fermentation to allow the buildup of biomass rapidly before diverting flux towards product synthesis. For a given batch time, there was an optimal switch time which maximized final product titers. This is intuitively clear when one realizes that shutting off growth too late would not give enough time for product synthesis; on the other hand, shutting off growth too early would not allow the buildup for enough biomass to be able to generate a high titer. Thus, the yield ([product]/[substrate]) and productivity ([product]/[batch time]) are conflicting goals and the strategy must be optimized such that both are high.

Computational simulations of potential implementation strategies have also been provided. A quorum-sensing based strategy was simulated to demonstrate that the acetate flux can be autonomously shut off during a fermentation to direct flux towards ethanol synthesis. The length of batch time affected the optimal switching time, which could be theoretically achieved by altering the synthesis of the chemical signal in the system. Similar strategies were also applied to simulate succinate and serine production.

1.4.2 Experimental Implementation of Dynamic Metabolic Engineering
Experimental implementation of dynamic strategies has also been actively pursued in direct pathway contexts. Farmer and Liao demonstrated one of the earliest examples of dynamic metabolic engineering by utilizing a sensor for acetyl-phosphate (ACP)\textsuperscript{19}. They showed that by placing lycopene production genes under the control of an ACP-responsive controller, they were able to increase lycopene titers by 18-fold and reduce acetate accumulation by nearly 3-fold. This was primarily because ACP accumulation is an indicator of excess glycolytic flux, and by turning on lycopene production in this regime, they were able to siphon carbon into product instead of acetate. This also improved growth significantly in the dynamic strategy compared to static expression of lycopene synthesis genes, some of which are inhibitory to growth. Moreover, Dahl et al utilized whole transcriptome analysis to identify promoters that respond to the toxic intermediate, farnesyl pyrophosphate (FPP), in the isoprenoid pathway\textsuperscript{20}. They used these promoters to control the upstream and downstream part of the pathway from FPP. Thus, accumulation of FPP shut off the upstream FPP biosynthesis genes, and turned on the downstream FPP-utilizing genes. This reduced protein costs, acetate accumulation and improved amorphadiene titers by nearly 2-fold. Similar sensor-based strategies have been implemented in other contexts to implement pathway-specific dynamic response\textsuperscript{21,22}.

While the aforementioned strategies have shown success in their respective contexts, they are limited to the scope of the metabolites and pathways for which they were developed. Thus, for each different pathway, the arduous task of devising and constructing a dynamic strategy would be required. It would first be necessary to determine whether the pathway at hand requires dynamic regulation, such as through quantitative metabolic or kinetic modeling. As most pathways, products, and intermediates do not necessarily have corresponding sensors for them, one would need to do omics-wide screening to identify elements, such as promoters or transcription factors, that respond to the metabolites of interest. Then, these novel elements would need to be cloned, characterized and tested for functionality. Often a large screen may yield tens to hundreds of candidates which must each be tested and characterized individually. One would then interface a subset of these elements with the pathway of interest to test performance and titers from the system. This cycle would need to be repeated for each different pathway, or variations of the same pathway, which produces slightly different metabolic contexts (such as different metabolite pools). Instead, a general pathway-independent strategy that can be applied to any pathway of interest is much more attractive as it would eliminate the endless cycle of reinvention of different dynamic strategies.

1.4.3 Implementations of Pathway-independent Regulation Strategies

One of the commonly proposed strategies revolve around developing medium formulation strategies that could allow cellular behavior to be dynamically controlled. “Autoinduction medium” is one formulation that varies the concentration of various sugars in the growth medium to trigger sugar-responsive pathways
and genetic parts in bacteria\textsuperscript{23}. If these parts are used to construct genetic circuits, one may dynamically modulate these circuits by modulating the external concentration of sugars in the medium. As sugars are used as feedstocks for fermentation processes, it would be advantageous to not tie their concentration to dynamic regulation strategies.

Previous work in the Prather lab has focused on building strategies for dynamically regulating heterologous pathways. The first attempts involved building manually controlled "valves" that can be used to siphon flux in desired directions at key branchpoints. Specifically, the utilization of glucose for growth or gluconate production was regulated using transcriptional regulation. In a PTS-deficient strain of \textit{E. coli}, glucose enters the cell through galactose permease (GalP) and is then phosphorylated by glucokinase (Glk) to be utilized in downstream glycolytic pathways. Gluconate is a model compound that is synthesized from free (unphosphorylated) glucose. Thus, a natural branchpoint occurs between the gluconate pathway and central carbon metabolism. Solomon et al devised a scheme whereby the transcription of Glk was shut off upon addition of an inducer (Figure 1A)\textsuperscript{24}. This led to a 20\% improvement in gluconate yield and a 70\% decrease in acetate yield. Strategies to impose valves based on translational control through anti-sense RNA were also investigated, but were found to be unsuccessful.

Post-translational control valves were also successfully implemented based on SspB-based protein degradation. For this system, the glucose-6-phosphate (G6P) node was chosen as a control point to siphon flux between central carbon metabolism and myo-inositol (MI) production\textsuperscript{25} (Figure 1B). Kinetic modeling was used to verify that degradation of phosphofructokinase (Pfk-1) would increase G6P pool sizes and thereby increase flux through the Inol pathway. In this system, addition of anhydrotetracycline (aTc) induced the production of SspB. Proteins, such as Pfk-1, which have been tagged with an SspB-dependent cleavage tag rapidly undergo degradation due to the action of ClpX and SspB\textsuperscript{26}. It was seen that even within one hour of aTc addition, the relative Pfk-1 activity had dropped nearly five-fold and caused growth arrest. A concomitant increase in G6P levels was also observed. An increase of \textasciitilde2-fold was seen in final MI titers after inducing SspB expression at 11.5 hrs after inoculation. Furthermore, it was seen that 11.5 hrs was the optimal time for SspB induction. Earlier or later induction times resulted in lower titers and yields. Overall,
this valve system was able to shut off essential metabolism at a certain point in the growth cycle and allow the carbon flux to instead be utilized for product formation.

![Diagram A](image1)

![Diagram B](image2)

**Figure 1:** (A) σTc-inducible transcriptional control of glucose uptake to dynamically control its flux between essential and heterologous pathways (B) σTc-inducible post-translational control at the G6P branchpoint to downregulate central carbon metabolism and increase flux into MI production

The next step in this sequence is to build strategies to autonomously switch the metabolic valve. Autonomous triggering of these flux-regulation modules, without the need for expensive inducers or human intervention, is critical to allow future adoption into commercial production processes, especially for commodity chemicals with low profit margins. Inducers, especially physiologically-active small molecules, are strictly prohibited in many bioprocesses as they add significant issues and costs in downstream processing. Although semi-autonomous, generalized strategies for dynamic flux regulation have been reported, no fully autonomous pathway-independent regulation modules have been constructed to systematically evaluate performance improvements and process robustness, with consideration for industrial applicability.

Quorum-sensing based strategies have been naturally the most common approach since they allow pathway-independent control, however, they have also relied on an initial addition of inducer to jumpstart the circuit. In the Prather lab, the Lux, Las and Rhl quorum sensing systems were tested in *Escherichia coli*. It was found, however, that they were difficult to implement due to the burden of expressing the synthases of the acyl-homoserine lactones (AHL), which are the key small molecules in quorum sensing. We wanted to
employ a different quorum sensing based system from *Panteoa stewartii* to implement a fully autonomous switching strategy that mitigates the need for inducers altogether. In addition we wanted to explore the versatility of this strategy in being utilized in different contexts and scales to understand its generality.

1.5 Industrial Applicability of Synthetic Biology Devices

While synthetic biology devices are rapidly being constructed in academic laboratory settings to actuate control in biological cells for industrial applications, the characterization necessary to make this very application possible are often left undone. In fact, recent literature demonstrated that since these cellular devices are often developed and tested in ideal, well-controlled conditions in the lab, any perturbation in the experimental conditions can rapidly change their performance\(^{34}\). Moser et al showed that the performance of circuits and their constituent parts varied notably across scales as they transitioned from 1 mL microtiter plates to shake flasks to 10 L bioreactors.

To consider translating devices and tools developed in controlled laboratory settings to industrial bioproduct processes, one must evaluate and understand their robustness as process conditions and configurations are varied. In our project, we sought to characterize the potential variability in performance which may result as we perturb the production conditions in which the developed synthetic biology strategies are evaluated.

1.6 Conclusions

Autonomous regulation of pathway fluxes has emerged as a new frontier in metabolic engineering\(^{35-37}\). The majority of previous studies associated with dynamic regulation have proposed strategies to sense and respond to specific intermediates or cell states\(^{20,21,38}\). While these strategies are effective and can generate interesting behaviors, such as metabolite oscillations, they are specific for the pathway or compounds for which they are developed. In this study, we propose strategies for dynamic flux control that may be generally applicable to all pathways, thereby striding into the next phase of metabolic engineering where complex genetic actuation during the course of fermentation can be achieved through "off-the-shelf" technologies and strains.

1.7 Thesis Aims and Objectives

Given the lack of relevant strategies, the goal of this thesis was to construct and characterize pathway-independent strategies to autonomously modulate metabolic fluxes, and to demonstrate improvement in production of corresponding products. More specifically, the aims of this thesis were as follows:
1) Construction and characterization of candidate modules for pathway-independent, autonomous control of enzymes in the context of a model pathway in *Escherichia coli*

2) Evaluation of robustness, scalability and extensibility to determine industrial applicability and usability

3) Building complexity to allow higher order *autonomous* control of pathways

### 1.8 Thesis Organization

This thesis is organized into five chapters. Chapter 1 provides the introduction, context and motivation of the work presented in this thesis. Chapter 2 discusses the construction of several pathway-independent strategies, and their successes and limitations when applied to the production of MI. One genetic circuit, which allowed dynamic transcriptional control of any target gene, appeared to be the most successful and versatile and was carried forward for further work for the remainder of the thesis. Chapter 3 describes the detailed evaluations performed to highlight the industrial applicability of the genetic device by evaluating its robustness to changes in culture media, culture scales as well as the pathways into which it was applied. Chapter 4 discusses our work in layering multiple devices to exercise multi-layer dynamic control over multiple genes in the same strain to further improve production of a desired compound, such as glucaric acid. Chapter 5 provides conclusions of the thesis work, as well as potential future directions.
Chapter 2

Construction of candidate modules for pathway-independent, autonomous control of pathway enzymes
Abstract

Autonomous dynamic modulation of metabolic pathway enzymes is a desired and necessary strategy as we move towards more complex products and pathways. Previous work has demonstrated that inducer-mediated genetic circuits that can dynamically curb glycolytic flux at intermediate points in fermentation can lead to improvements in titers of myo-inositol (MI) and glucaric acid in *E. coli*. We constructed and evaluated different strategies to institute autonomous dynamic control of gene expression, especially of phosphofructokinase-1 (Pfk-1) from upper glycolysis. A quorum sensing-based circuit to transcriptionally downregulate expression of a target gene was used to control Pfk-1 expression. This circuit was fully autonomous and did not require any exogenous inducer addition, as the quorum sensing signaling molecule was synthesized by the cognate synthase that was constitutively expressed from the genome. Varying the expression level of the synthase allowed variation in the production rate of the signaling molecule, and thereby a modulation of the switching time of the circuit. A library of strains was created that had differential expression level of the synthase and thereby different circuit switching times. Using this library, a large space of switching times was evaluated to knockdown glycolytic flux. A 20% boost in MI titers was achieved with the best strain that contained an intermediate switching rate. Other strategies and circuits based on nutrient limitation and post-translational downregulation were also evaluated.

This chapter contains material adapted from:

2.1 Introduction

We sought to build strategies that could be used to dynamically modulate metabolic fluxes in host cells in an autonomous fashion. Theoretically, such strategies could be utilized to temporally control the flux down various branches of complex metabolic networks to optimize titer of the final product. Numerous scenarios may be conceptualized where dynamic metabolic engineering would be advantageous. Metabolic branchpoints where a common metabolite can either be siphoned into native growth pathways or the heterologous product formation pathway is one scenario where optimal dynamic regulation of flux between the two branches may improve titers over static strategies. The Prather lab has investigated this scenario in the context of glucaric acid production, in which glucose-6-phosphate (G6P) may be directed either into glycolysis for essential cell functions, or be converted to myo-inositol (MI) through myo-inositol-1-phosphate synthase (INO1), as shown in Figure 2. MI can be further converted into other useful products, such as glucaric acid, a biopolymer precursor, and scylo-inositol, which has been studied as a therapeutic for Alzheimer’s. The pathway for glucaric acid has been engineered in E. coli and theoretical yields of almost 100% are possible; however, G6P is directed into this pathway at the expense of central carbon metabolism. The relative kinetic efficiency between the competing enzymatic branches determines the split of G6P flux in wild-type strains, as well as the potential improvement in MI and glucaric acid titers with dynamic down-regulation of native metabolic flux. Previous studies in the Prather lab have demonstrated that shutting off glycolytic flux by downregulating phosphofructokinase-1 (Pfk-1) at intermediate points during the fermentation leads to increased G6P pools, and redirection of incoming carbon towards production of MI and glucaric acids. Such dynamic flux regulation was achieved by installing a circuit that may be induced by anhydrotetracycline (aTc) to degrade cellular Pfk-1. Addition of aTc at various
times over the course of the fermentation allowed identification of the optimal time to maximally boost titers of MI and glucaric acid.

We sought to create circuits that could be used to autonomously modulate pathway fluxes without the need to manually add inducers. Autonomous triggering of these flux-regulation modules, without the need for expensive inducers or human intervention, is critical to allow future adoption into commercial production processes, especially for commodity chemicals with low profit margins\textsuperscript{27}. Inducers, especially physiologically-active small molecules, are strictly prohibited in many bioprocesses as they add significant issues and costs in downstream processing\textsuperscript{28}.

One strategy that has been previously proposed for autonomous circuit triggering has been to utilize circuit parts that can respond to concentrations of certain components of the growth medium. Modulating the concentration of sugar sources in the medium has been one strategy\textsuperscript{21} to induce certain desired cell behaviors. Since we specifically wanted to utilize our dynamic device to modulate carbon flux coming from external sugar sources, we chose to utilize a more orthogonal nutrient source to act as circuit inducer – phosphate. Circuit parts that are activated upon depletion of phosphate were utilized to trigger degradation of Pfk-1, such that the time of triggering can be modulated by varying the starting phosphate concentration in the medium. This strategy, however, revealed that global metabolic effects due to depletion of essential nutrients can confound circuit performance, thereby limiting industrial applicability.

As an alternative, quorum sensing (QS) systems are a well-known mechanism for executing cell-density dependent processes, and have been used synthetically for applications such as timed induction of recombinant proteins\textsuperscript{30}, timed lysis\textsuperscript{42}, and balancing of different cell populations\textsuperscript{43}. QS involves the accumulation of small molecule signals which then lead to the induction of the QS circuit and the genes it controls. We utilized parts of the Esa QS system, originally derived from \textit{Pantoea stewartii}\textsuperscript{44}, to create circuit variants that autonomously switch off gene expression at desired times and cell densities. These circuits were genomically integrated in \textit{Escherichia coli} and were used to dynamically control endogenous genes involved in essential pathways. As a first demonstration, we sought to install a QS circuit to dynamically downregulate Pfk-1 and glycolytic flux to siphon carbon into MI production. After an initial “growth” phase to allow biomass accumulation, the QS-based circuit switched the culture to “production” mode. Using this system in \textit{E. coli}, we were able to achieve increases in both titer and yield of MI, a precursor in glucaric acid production.

\subsection*{2.2 Materials and Methods}
All strains and plasmids used in this study are summarized in Table 1. For plasmid preparations and genetic manipulations, cells were cultured in Luria-Bertani (LB) broth at either 30 °C or 37 °C. Temperature-sensitive plasmids were cured at 42 °C. Strain IB1379 was previously constructed.

2.2.1 Strain construction

2.2.1.1 QS-based transcriptional control of GFP

For initial characterization of QS-based transcriptional control with varying switching times, the esarI70V expression cassette was integrated into the genome under the control of a synthetic promoter (apFAB104)\textsuperscript{45}. esarI70V is a mutant version of the wildtype esar that was previously engineered to respond to higher threshold concentrations of AHL than the wildtype protein\textsuperscript{46}. Integration was performed via "clonetegration"\textsuperscript{47}. The pOSIP-KH backbone and the desired EsaRI70V expression cassette were digested with Kpnl and PstI and ligated overnight. The ligation product was used to transform E.coli strain MG1655 for integration at the HK022 locus. The phage integration genes and antibiotic resistance cassette were cured with pE-FLP\textsuperscript{47}, yielding strain AG2681. The Esar expression library was installed as described below.

The Pesas promoter was amplified from pCS-PesaS-lux\textsuperscript{46} and was appended to GFP tagged with a modified SsrA tag (AANDENYALVA, “LVA”) using overlap extension PCR to yield \( P_{esas} \)-GFP(LVA). This cassette was inserted into the pCOLA Duet Vector using restriction digestion and ligation to yield pCOLA-Pesas-GFP(LVA) (Table 1). A separate version of this plasmid was also created in which GFP was not appended to any degradation tag, yielding pCOLA-Pesas-GFP (Table 1).

2.2.1.2 QS-based transcriptional control of Pfk-1

For QS-based transcriptional control of Pfk-1, the pfkA locus was amplified from the E. coli genome with primers that appended the standard SsrA degradation tag (AADENYALAA, “LAA”). The \( P_{esas} \) promoter was appended to pfkA using overlap extension PCR to yield \( P_{esas} \)-pfkA(LAA). This cassette was used to replace the native pfkA locus and the corresponding promoter using the "landing pad" method\textsuperscript{48}. Briefly, this cassette was cloned into vector pTKIP-neo by restriction digestion and ligation with HindIII and Kpnl, yielding pTKIP-Pesas-pfkA(LAA). Lambda-red mediated recombination was used to introduce the tetracycline resistance marker and "landing pad" sequences into the genome at the pfkA locus in IB1379. This strain was transformed with pTKRED and pTKIP-Pesas-pfkA(LAA) and integration was achieved after cleavage of the pTKIP-Pesas-pfkA(LAA) plasmid and subsequent lambda-red-mediated recombination of the \( P_{esas} \)-pfkA(LAA) and kanamycin resistance cassette sequences into the pfkA locus. The kanamycin resistance cassette was cured by expression of FLP recombinase from pCP20. Next, the esarI70V was integrated into...
the genome under control of a synthetic promoter (apFAB104) at the HK022 phage locus using "clonetegration"\textsuperscript{47}, as described previously, yielding strain IB2275.

2.2.1.3 QS-based post-translational control of Pjk-1

To construct strains that utilize QS-based induction of sspB, an EsaR-responsive expression cassette for sspB expression was first constructed. Briefly, esaR was amplified from pAC-EsaR\textsuperscript{46} using primers that appended the 5' UTR and a synthetic promoter (apFAB104). Additionally, P_{esaR} was amplified from pCS-P_{esaR}-lux\textsuperscript{46}, and sspB was amplified from the \textit{E. coli} genome. These three pieces were joined together using overlap-extension PCR to create esaR-P_{esaR}-sspB. The cassette was cloned into pOSIP-CH\textsuperscript{47} using restriction digest with PstI and KpnI, and the resultant construct was used to transform \textit{IB1643} to allow genomic integration at the HK022 locus. Phage integration genes and the antibiotic resistance marker were cured with pE-FLP as described previously\textsuperscript{47} to yield strain IB2265.

To reduce the translation rate of sspB, a genomic RBS library with varying levels of predicted translational efficiency was inserted into the chromosome using methods described in Reisch et al\textsuperscript{49}. Briefly, we first generated a library of RBS variants of different strengths by using the RBS calculator\textsuperscript{50} and introducing a three base pair degeneracy into the RBS and 5' UTR of sspB in IB2265. An 80-mer oligonucleotide that targeted the lagging strand of the sspB RBS (\texttt{gttaagtaaagaagcagccaattcatt\underline{aagaaag}N\textit{N}gaaaggatcNatggtttgctacagctacaccacgctgctcct}) that contained three nucleotide degeneracies (denoted as "N" in the sequence above) was obtained. The underlined portion in the sequence denotes the original RBS sequence. In addition, a guide RNA sequence was designed to target a 21-base pair region (\texttt{ttaaagagagaaggatcc}) that was adjacent to an NGG sequence in the original RBS sequence to allow Cas9 counterselection against the wild-type genome. Strain IB2265 was first transformed with plasmid pCas9CR, for Cas9 expression, and then with pKDsgRNA-sspB, which possessed the lambda-Red genes in addition to the sgRNA. The lambda-Red genes were expressed by the addition of 1% arabinose and incubated for 15 minutes and then transformed with the 80-mer. After a two-hour recovery, the cells were plated on anhydrotetracycline-containing medium to induce Cas9 counterselection. Colonies were screened in the BioLector (m2plabs, Baesweiler, Germany) for growth with and without AHL in MOPS minimal medium (described below), and one was chosen for further characterization, yielding strain AG2349.

2.2.1.4 EsaI expression library

To implement a library of promoter and RBS variants to drive esaI expression from the genome, a select number of promoters and RBS variants were chosen whose combinatorial assembly spanned the predicted
expression space in Mutalik et al\textsuperscript{51}. We chose five promoters and six RBS variants to give a total library size of 30 combinations. A comprehensive list of all combinations that were constructed for the Esal expression library and the associated expression strengths is provided in Table 2. A single RBS variant from the BioFAB registry was used as a template for PCR amplification with primers containing the different promoters at the 5' end, and the variable regions of the RBS variants at the 3' end. This led to a series of amplicons containing different promoter and RBS variant combinations. The *esal* locus was amplified from pAC-EsaRI\textsuperscript{52} and was appended to the amplicons containing promoters and RBS variants using overlap extension PCR. The combined fragments were cloned into pOSIP-KO\textsuperscript{47} using restriction digestion with KpnI and SphI, and AG2681, IB2275 and AG2310 were transformed with the ligated plasmids to yield a library of strains with *esal* genomically integrated at the 186 phage locus. The complete list of all combinations of Esal cassettes and the corresponding strain denotations are given in Table 2. Strains based on post-translational knockdown of Pfk-1 were only prepared with a subset of the Esal library.

2.2.1.5 Phosphate-starvation-based control

A list of all strains utilized in this study and the relevant genotypes are listed in Table 1. Strains IB1863, IB1624, IB1643, and IB1379 were previously constructed\textsuperscript{23}. To engineer strains that induce *sspB* upon phosphate depletion, the *PphoA* promoter and a synthetic 5' UTR were appended to *sspB* using an extended 5' overhang on the PCR primer for amplification of the *sspB* sequence from the *E. coli* genome. Integration of the *sspB* expression cassette into the genome was carried out via “clonetegration”. The pOSIP-CH backbone and the desired *sspB* expression cassette were digested with KpnI and PstI and ligated overnight. The ligation product was used to transform strains IB1624 and IB1643 for integration at the HK022 locus. The phage integration genes and antibiotic resistance cassette were cured with pE-FLP, yielding the strains IB1509 and IB643.

2.2.2 Culturing and fermentations

2.2.2.1 GFP characterization

Switching times for the various Esal variants were quantified using the BioLector microbioreactor system (m2p-labs, Baesweiler, Germany). Individual colonies were inoculated in LB medium and grown overnight at 30 °C. Working 1-mL cultures were inoculated from these seeds into BioLector 48-well flower plates and incubated at 30 °C, 1200 rpm (3 mm orbit), and 80% relative humidity. The plate was sealed with gas-permeable sealing foil (m2p-labs) to reduce evaporation. Cultures were monitored for OD (BioLector Units) and GFP fluorescence over time. To ascertain switching ODs for a given strain, the corresponding cell density at the point of peak fluorescence was obtained from the fluorescence and biomass time curves.
It was assumed that higher AHL production rate and stronger EsaL expression would lead to lower switching ODs. Strains were rank-ordered based on switching ODs and the trends were compared to the rank-ordered list of strains based on predictions from Mutalik et al.21

2.2.2.2 Growth-based screening

The RBS libraries generated to tune down the translation rate of sspB were screened for growth using the BioLector microbioreactor system (m2p-labs, Baesweiler, Germany). Individual colonies were inoculated in LB medium, grown overnight at 30 °C at 250 rpm, then diluted 1:100 into modified MOPS medium and grown for ~24 hrs. Working cultures of 1 ml total volume in MOPS medium were inoculated from this seed in a BioLector 48-well flower plate and incubated at 30 °C, 1200 rpm (3 mm orbit), and 80% relative humidity. Each seed culture was used to inoculate two wells in the BioLector plate - one supplemented with 10 µM AHL and one without AHL. The plate was sealed with gas-permeable sealing foil (m2p-labs) to reduce evaporation. Culture OD was monitored over time.

2.2.2.3 Shake flask fermentations

Initial growth studies and MI production trials were performed in 250-ml baffled shake flasks with 60-ml working volume at 30 °C and 250 rpm in modified MOPS minimal medium containing 10 g/L D-glucose, 3 g/L NH₄Cl, 1 g/L K₂HPO₄, 2 mM MgSO₄, 0.1 mM CaCl₂, 40 mM MOPS, 4 mM Tricine, 50 mM NaCl, 100 mM Bis-Tris, 134 µM EDTA, 31 µM FeCl₃, 6.2 µM ZnCl₂, 0.76 µM CuCl₂, 0.42 µM CoCl₂, 1.62 µM H₃BO₃, and 0.081 µM MnCl₂. When noted, the medium was also supplemented with 0.2% casamino acids. For phosphate starvation experiments, the concentration of K₂HPO₄ in the medium was varied from 0.1 to 1 g/L. For strains containing pTrc-INO1, the medium was also supplemented with 100 µg/ml carbenicillin for plasmid maintenance. Strains were initially grown in 3-ml LB medium at 30 °C overnight, and then diluted 1:100 into 5-ml seed cultures of modified MOPS medium (containing 0.2% casamino acids if the subsequent fermentations were also performed with casamino acids) for ~24 hrs at 30 °C. These were used to inoculate working flask cultures at OD₆₀₀ 0.05. For MI production trials, shake flask cultures were also supplemented with 50 µM β-D-1-thiogalactopyranoside (IPTG) to induce INO1. Flasks were incubated at 30 °C with 250 rpm shaking and 80% humidity. Samples were taken periodically for measurement of enzyme activity, mRNA quantification, and extracellular metabolites. Fermentations were carried out for 72 or 95 hrs, at which point all glucose was consumed.

2.2.3 Phosphofructokinase activity assays

Samples for activity assays of Pfk-1 were collected over the course of the fermentations. Activity measurements were carried out according to the protocol reported previously.25
All enzymatic activity assays were carried out on crude lysates. For preparation of lysates, samples of 5-10 ml of cell culture were collected, frozen at -80°C, and then resuspended in 50 mM Tris-HCl, pH 7.4 (0.25 – 1 ml, depending on cell density). Cells were lysed via bead beating for 5 minutes and lysates clarified by centrifugation at 15,000 x g for 15 minutes. The assay mixture consisted of 0.1 M Tris – HCl (pH 8.2), 10 mM MgCl₂, 1 mM ATP, 0.2 mM β-NADH, 1 mM fructose-6-phosphate (F6P), 1 mM NH₄Cl, 0.01% Triton X-100, 0.83 U aldolase, 0.42 U triosephosphate isomerase, and 0.42 U glycerophosphate dehydrogenase. Reaction progress was followed by measurement of absorbance at 340 nm. One unit of Pfk activity was defined as the amount required to convert 1.0 µmole of ATP and D-fructose 6-phosphate to ADP and fructose 1,6-bisphosphate per minute at pH 8.2 and room temperature.

2.2.4 Quantification of mRNA levels

To quantify the relative transcript levels of pfkA across various strains, 1-ml samples were collected over the course of the fermentation and mixed with 2-ml of RNAprotect bacteria reagent (Qiagen). These were vortexed briefly to mix, incubated for 5 min at room temperature, centrifuged at 5000xg for 10 min to pellet the cells and stored at -80 °C until processing.

Total mRNA was extracted using the illustra RNAspin Mini Kit (GE Healthcare Life Sciences). The eluted total RNA was subjected to an additional DNasel digestion step to remove genomic DNA using the TURBO DNA-free™ Kit (Life Technologies). Concentrations were measured using Nanodrop 2000 (Thermo Scientific). Next, the cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen), ensuring that equal amounts of RNA was added to every reaction for normalization across samples. Protocols were followed as provided by the respective kits. Quantitative PCR was performed using the ABI 7300 Real Time PCR System (Applied Biosystems), and the 2X Brilliant II SYBR qPCR High ROX Master Mix (Agilent Technologies). Reactions contained 2 µL of cDNA from the corresponding RNA isolation, as well as appropriate primers and the master mix quantities as described by the protocols provided with the kit to give a total reaction volume of 25 µL. Amplification was carried out using the following program: initial incubation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 1 min. Threshold cycle values were set by the software and the CT values for each respective sample were obtained and used to calculate fold-differences in pfkA transcript levels compared to IB2275.

2.2.5 Quantification of extracellular metabolites

Glucose, MI, acetate and glucaric acid levels were quantified by high performance liquid chromatography (HPLC) on an Agilent 1100 or 1200 series instrument (Santa Clara, CA) with an Aminex HPX-87H column (300 mm by 7.8 mm; Bio-Rad Laboratories, Hercules, CA). Sulfuric acid (5 mM) at a flow rate of 0.6
mL/min (isocratic) was used as the mobile phase. Compounds were quantified from 10 μl sample injections using a refractive index detector. Column and refractive index detector temperatures were held at 55° C for MI quantification.
Table 1: Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>QS-based transcriptional control of GFP expression (Pesas)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AG2681</td>
<td>MG1655 HK022::apFAB104-EsaR70V</td>
<td>EsaR70V under control of constitutive promoter</td>
</tr>
<tr>
<td>LXX</td>
<td>MG1655 HK022::apFAB104-EsaR70V 186(O)::apFABXX-apFABXX-esal</td>
<td>AG2681 + Esal driven by a specific promoter and RBS, generalized as &quot;apFABXX&quot; here. The full list of promoter-RBS combinations is given in Supplementary Table 1. For GFP characterization (Fig. 1B and 1C), this strain series was transformed with pCOLA-Pesas-GFP(LVA)</td>
</tr>
<tr>
<td><strong>QS-based transcriptional control for metabolite production (Pesas)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IB1379</td>
<td>MG1655 ΔendΔΔzwf ΔpfkB</td>
<td>Wildtype pfkA promoter control</td>
</tr>
<tr>
<td>IB2275</td>
<td>MG1655 ΔendΔΔzwf ΔpfkB pfkA::Pesis-pfkA(LAA) HK022::104-esar170V</td>
<td>Transcriptional control of tagged pfkA under Pesas promoter; esaR170V under control of a synthetic promoter (apFAB104)</td>
</tr>
<tr>
<td>L24S</td>
<td>MG1655 ΔendΔΔzwf ΔpfkB pfkA::Pesis-pfkA(LAA) HK022::104-esar170V 186(O)::apFAB295-apFAB699-esal</td>
<td>IB2275 + esal driven by selected promoter and RBS sequences from Mutalik et al; Predicted strength: 4.8%</td>
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<td>L30S</td>
<td>MG1655 ΔendΔΔzwf ΔpfkB pfkA::Pesis-pfkA(LAA) HK022::104-esar170V 186(O)::apFAB65-apFAB699-esal</td>
<td>See description for L24S; Predicted strength: 2.9%</td>
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<td>L19S</td>
<td>MG1655 ΔendΔΔzwf ΔpfkB pfkA::Pesis-pfkA(LAA) HK022::104-esar170V 186(O)::apFAB295-apFAB700-esal</td>
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<td>See description for L24S; Predicted strength: 1.3%</td>
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<td>L31S</td>
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<td>See description for L24S; Predicted strength: 1.2%</td>
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**Phosphate-limited transcriptional control (PphoA)**
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<th>Plasmid</th>
<th>Functional Genotype</th>
<th>Source</th>
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<tr>
<td>pCOLA-PesaS-GFP(LVA)</td>
<td>Tagged GFP under the ( \text{P}_{\text{esaR}} ) promoter in the pCOLA DUET vector</td>
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<tr>
<td>pCOLA-PesaS-GFP</td>
<td>GFP under the ( \text{P}_{\text{esaR}} ) promoter in the pCOLA DUET vector</td>
<td>This study</td>
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<tr>
<td>Vector</td>
<td>Description</td>
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<td>pTrc-INO1</td>
<td>pTrc99A with <em>S. cerevisiae</em> INO1</td>
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<tr>
<td>pRSFD-IN-MI</td>
<td>INO1 (<em>S. cerevisiae</em>) and MIOX (<em>M. musculus</em>) under T7 promoter</td>
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<tr>
<td>pTrc-udh</td>
<td>Udh (<em>P. syringae</em>) expressed under the Trc promoter</td>
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<tr>
<td>pAC-EsaR</td>
<td>p15A; wildtype EsaR variant</td>
<td>46</td>
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<tr>
<td>pAC-EsaRI</td>
<td>p15A, EsaR and EsaI operon</td>
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<tr>
<td>pCS-Pesa-lux</td>
<td>Pesas inserted to drive lux operon</td>
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<tr>
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<td>Pesas inserted to drive lux operon</td>
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<tr>
<td>pOSIP-KH</td>
<td>attP HK022, ccdB, HK022 integrase expressed by λ pR under control of λ c1857</td>
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<td>pE-FLP</td>
<td>FLP recombinase expressed by pE</td>
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<td>pCas9CR</td>
<td>Cas9 expression</td>
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<tr>
<td>pKDsgRNA-sspB</td>
<td>Guide RNA targeting 20-bp sequence of original sspB RBS; Prapa λR λB λexo</td>
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<td>pTKIP-neo</td>
<td>ColE1(pBR322)</td>
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<tr>
<td>pTKRED</td>
<td>repA101ts, araC, Prara λR λB λexo, lacI, Prara - 1-SceI</td>
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<tr>
<td>pCP20</td>
<td>FLP recombinase expressed by λ pR under control of λ c1857</td>
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Table 2: All combinations of promoter and RBS variants constructed to yield a library of EsaI expression levels. Part numbers denote those registered in the BioFAB database. Predicted strengths of the combinations are represented as a percentage of the maximal strength found by Mutalik et al.\(^5\). We were unable to integrate L2 and L17.

<table>
<thead>
<tr>
<th>Promoter</th>
<th>RBS</th>
<th>Denotation</th>
<th>Predicted strength of EsaI expression(^5)</th>
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<td>apFAB682</td>
<td>L1</td>
<td>66.6</td>
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<td>apFAB683</td>
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2.3 Results

2.3.1 Instituting Phosphate-Starvation-based triggering of Pfk-1 downregulation

A previous configuration for dynamic downregulation of Pfk-1 activity relied upon the appendage of an SspB-dependent SsrA tag, and required manual addition of inducers to initiate production of SspB to recruit ClpXP to the tagged protein and trigger active degradation\(^\text{5}\). In this new architecture, the promoter driving sspB expression in the genome was replaced with one that is activated upon depletion of phosphate (\(P_{phoA}\))\(^\text{54}\) to allow autonomous induction of sspB and post-translational knockdown of Pfk-1 activity in a zwf knockout strain upon phosphate depletion (Figure 3A). The pfkA sequence was appended to an SspB-dependent degradation tag (AADENYSENYADAS, "DAS+4")\(^\text{26}\), and was inserted in the genome under a constitutive promoter from the BioFAB registry (apFAB114 or apFAB104), yielding strains IB643 and IB1509 (Table 1). In MOPS minimal medium with glucose as the sole carbon source, depletion of phosphate led to induction of sspB, resulting in rapid degradation of Pfk-1 protein in the cell, arrest of cell growth and redirection of flux into MI production. This was compared against a strain where sspB expression was driven by an aTc-inducible promoter (IB1863, Table 1), but was not induced.

Upon phosphate starvation, IB1863 showed no decrease in Pfk-1 activity, while both IB643 and IB1509 showed decreases (Figure 3B). The baseline activity under excess phosphate revealed that IB643 had a much lower basal Pfk-1 activity than either IB1863 or IB1509. Thus, we decided to proceed with IB1509 for MI production as it more closely matched the control. Both strains showed a boost in titers in phosphate-limiting conditions (Figure 3C) if growth was arrested at an intermediate point with phosphate depletion. With initial phosphate levels too low (highly phosphate-limited), sufficient biomass could not be formed and glucose was not fully depleted. Thus, a higher phosphate concentration (0.2 g/L) still allowed phosphate depletion in the culture at a later time while allowing more biomass to build up. Interestingly, we observed that even in the IB1863 control, a boost in MI titers was observed under phosphate-limiting conditions and was not discernible from that obtained from IB1509 (Figure 3C). The reasons for this have not yet been fully investigated, but could include natural limitation of glycolytic flux during phosphate starvation or upregulation of endogenous phosphatases that dephosphorylate myo-inositol-1-phosphate to MI more rapidly, helping to pull flux through INO1.
The use of phosphate starvation to drive \textit{sspB} expression, and correspondingly drive a reduction in Pfk-I activity, is an interesting strategy for a number of reasons. Phosphate is an essential nutrient and phosphate starvation strategies have already been demonstrated to improve yields for a number of products, such as shikimic acid and fatty acids\textsuperscript{55,56}. Additionally, phosphate feeding could be used to achieve cycling in the Pfk-I activity level, unlike in the case of inducer addition, where once added, the inducer cannot be easily removed to stop \textit{sspB} expression and allow recovery of Pfk-I activity. However, nutrient starvation also induces a wide variety of regulatory responses in the cell, some of which may be undesirable for product formation. Furthermore, linking nutrient starvation with a specific, desired outcome could provide a route to enhance positive effects on production, but it may still be difficult to achieve general applicability for different pathways and products, as seen by our results above. This strategy also relies specifically on the medium composition, such that a precise amount of phosphate must be present in order to enable switching at desired times. This may be especially undesirable for industrial production where media formulations may be tailored to the pathway and products of interest or may include complex feedstocks and other

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**Figure 3. Phosphate starvation-based control of \textit{sspB} expression and Pfk-I activity.**

(a) Schematic of the phosphate starvation-based circuit controlling \textit{sspB}. (b) IB643 and IB1509 contained identical strain backgrounds except for the strength of the constitutive promoter driving \textit{pfkA} expression. Depletion of phosphate led to a decline in Pfk-I activity in IB643 and IB1509. The latter was chosen for production trials as its basal activity in excess phosphate more closely matched the IB1863 control. (c) Production of MI with different initial phosphate levels. There was no significant difference in titers between IB1863 and IB1509. Both stains showed improved production at intermediate phosphate levels, compared to very low or excess phosphate. Error represented as s.d of triplicate trials.
components for more economical process operation\textsuperscript{57,58}. This limitation extends to all nutrient-based strategies as they rely on the careful manipulation and monitoring of medium recipes.

2.3.2 QS-based strategies to exercise dynamic control of gene expression

As limitations of nutrient-based strategies became more apparent, we sought strategies which may be more orthogonal to host metabolism and may have less undesired compound effects that could confound strain performance. Quorum sensing (QS) circuitry provided a plausible alternative that may be more self-contained and may interfere less with host metabolic pathways. Quorum sensing is a bacterial cell-cell communication mechanism that relies on the cell-density dependent production and sensing of small molecule signals\textsuperscript{59}. As bacterial cells grow and divide, they produce small molecule signals, such as acyl-homoserine lactones specific to the quorum sensing network present in that strain. As the extracellular density of these signals increases in the culture, it reaches a threshold beyond which it elicits specific behaviors, such as bioluminescence\textsuperscript{60} and virulence\textsuperscript{61-63}. Some of the most commonly engineered quorum sensing systems include the Lux, Las and Rhl systems from \textit{Vibrio cholerae} and \textit{Pseudomonas aeruginosa}\textsuperscript{64}. As previous work in the Prather lab showed that these systems cause severe burden on the host cell\textsuperscript{64}, we decided to utilize a different, less-utilized quorum sensing system.

The Esa system from \textit{Pantoea stewartii} is similar to the Lux system and utilizes the same signaling molecule, 3-oxohexanoyl-homoserine-lactone (3OCH\textsubscript{6}HSL, denoted from here on as “AHL”\textsuperscript{44}). The system consists of three major parts: the regulator (EsaR), the AHL synthase (Esal) and the EsaR-binding promoter (P\textsubscript{esar} or P\textsubscript{esas}). In the absence of AHL, EsaR is bound to DNA\textsuperscript{44}. The P\textsubscript{esas} promoter is activated by EsaR binding, while the P\textsubscript{esar} promoter is repressed by EsaR binding (Figure 4). The Collins lab at Rensselaer Polytechnic Institute has done additional work to characterize and import this system into \textit{E. coli}. Specifically, they have used directed evolution to evolve variants of EsaR that have varying affinities for the cognate AHL\textsuperscript{46}. In addition, they have also engineered the P\textsubscript{esar} and P\textsubscript{esas} promoters to have different binding affinity towards EsaR\textsuperscript{52}. Consequently, they have been able to show that variations of the system utilizing these new parts allow the system to respond to varying AHL thresholds. Due to the potential versatility of this system in providing different timing for response, we have utilized the Esa system and its engineered parts to create a pathway-independent regulation system. Specifically, we have utilized both the wildtype and the EsaRI70V variant of EsaR. The latter variant is less sensitive to AHL than the wildtype EsaR variant and therefore requires a higher AHL concentration to unbind from DNA. The identity of the
variant used in each instance is indicated correspondingly. We also used the wildtype \( P_{\text{esAR}} \) and \( P_{\text{esAS}} \) promoters for the remainder of this thesis.

![Diagram](image)

**Figure 4:** EsaR binds to DNA in the absence of AHL and (A) represses expression from the \( P_{\text{esAR}} \) promoter and (B) activates expression from the \( P_{\text{esAS}} \) promoter. AHL interaction with EsaR allows it to unbind and (A) activate expression from \( P_{\text{esAR}} \) and (B) deactivate expression from \( P_{\text{esAS}} \).

2.3.2.1 Mathematical Simulation of QS-based knockdown of Pfk-1 for MI production

Computational justification for dynamic switching between growth and production modes has been demonstrated for several products\(^{16,17}\). In the case of MI and glucaric acid production, the relative kinetic competition between the enzymatic branches utilizing G6P pools determines any advantages that may be achieved by autonomously and dynamically balancing tradeoffs between cell growth and product formation. A theoretical model was constructed using published *in vitro* kinetic data and information on AHL production dynamics to simulate a QS-based autonomous valve that downregulates Pfk-1 expression and glycolytic flux during the course of the fermentation (model details provided in Appendix 1). G6P pools are sourced through glucose uptake and depleted through utilization by INO1 and glycolysis (Pgi and Pfk-1), in a strain background where *zwf* has been deleted to prevent flux into the pentose phosphate pathway. The temporal flux profile through INO1 and endpoint MI titers were calculated to determine the benefits of autonomous redirection of metabolite flux.

As biomass and AHL accumulate over the course of the fermentation, the cellular Pfk-1 activity (expressed as "Fractional Pfk-1") continuously declines, resulting in increased G6P pools and higher flux through INO1. Assuming glucose is the sole carbon source in defined culture medium, the decrease in Pfk-1 activity feeds back to proportionally decrease growth rate, as well as affect cell-density dependent production of AHL. A higher AHL production rate leads to faster AHL buildup, and an earlier decline in Pfk-1 activity and onset of growth arrest, signifying faster switching from "growth" to "production" mode (Figure 5A). The rate of restriction of glycolysis and growth, and redirection of G6P flux into MI production varied as a function of AHL production rate. Endpoint MI titers were predicted based on integration of both the
temporal flux of INO1 and biomass profiles in the culture (Figure 5B). MI titers peak at an intermediate AHL production rate, representing a 1.6-fold boost over the static case where Pfk-1 activity is not controlled. An optimal production rate is predicted, which balances growth in the culture with the length of "production" mode, such that a boost in MI titers is obtained. If knockdown in Pfk-1 activity occurs too fast, titers are suboptimal as growth arrest occurs too early and biomass levels are too low. On the other hand, if knockdown in Pfk-1 occurs too late, the duration of the "production" mode is too short. The model thus indicates that experimentation with a spectrum of AHL production rates is necessary to optimize the system.

![Graph](image)

**Figure 5.** Theoretical simulations of QS-based dynamic downregulation of Pfk-1 to ascertain expected trends in balancing growth and MI production from the G6P branchpoint. (a) A "1x" cellular AHL production rate was initially set to that used in You et al.\(^6\) (1.3 \times 10^{-10} \text{ nM ml s}^{-1}) and then varied accordingly. Lower AHL production rate leads to slower AHL accumulation and downregulation of Pfk-1 activity, and later growth arrest. The "static" trajectory refers to a strain without dynamic downregulation of Pfk-1. Fractional Pfk-1 denotes the Pfk-1 activity in the cell as a fraction of the basal levels without any downregulation. Kinetic parameters and rate equations are summarized in Table 7. (b) Integration of the biomass and INO1 flux profiles over the batch time yielded expected endpoint MI titers for given AHL production rates. Titer profile shows an optimal AHL production rate to maximize titers, such that a very fast or very slow Pfk-1 knockdown is suboptimal. The dashed line indicates the corresponding expected MI titer from the "static" strain.

2.3.2.2 Post-translational control of gene expression through the PeaR architecture

The EsaR-repression architecture was utilized to induce post-translational degradation of cellular Pfk-1 protein (Figure 6). Specifically, the pfkA sequence in the genome was appended to a modified SsrA tag (AAADNYSEN YADAS, "DAS+4")\(^{26}\) that induces rapid protein degradation in the presence of SspB, a protein that recruits ClpXP to the tagged site. This tagged pfkA was inserted into its native locus under the
control of a constitutive promoter from the BioFAB registry (apFAB114). An esaR-PesoR-sspB cassette was separately integrated into the genome in which esaR was driven by a constitutive BioFAB promoter (apFAB104), while sspB was under the control of the PesoR promoter. Flux into the pentose phosphate pathway was arrested by knocking out zwf, yielding strain IB2265 (Table 1). In the absence of AHL, EsaR is bound to the PesoR promoter and prevents production of SspB. The control IB1643 strain, which contained tagged pfkA under the same promoter as IB2265 but with no esaR-PesoR-sspB expression cassette, exhibited no decline in Pfk-1 activity with or without AHL (Figure 7A). On the other hand, IB2265 exhibited low Pfk-1 activity, even in the absence of any AHL. Exogenous addition of AHL led to no further decline in activity, suggesting that SspB may already be present due to leaky expression from the PesoR promoter. Furthermore, in minimal medium with glucose as the sole carbon source, this strain exhibited no growth in the presence or absence of AHL (Figure 7B). This suggested that there may be sufficient leaky expression of SspB to downregulate Pfk-1 activity, despite no AHL addition.

In order to reduce basal leaky expression of sspB, the strength of the RBS controlling its translation was tuned down directly within the genome through insertion of a degenerate base-pair library and Cas9-based counterselection\textsuperscript{49,50}. This led to a spectrum of strains containing RBS variants of different strengths. Colonies were screened for growth on MOPS minimal medium with glucose as the sole carbon source, in the presence and absence of exogenously added AHL using the BioLector microbioreactor system. Several colonies were isolated that showed normal and no growth in the absence and presence of added AHL, respectively (Figure 7B). One was then chosen that had the weakest predicted RBS strength (AG2349) and was used for further characterization and production trials.

esal was genomically-integrated into AG2349 under the control of the Esal promoter-RBS library to yield a spectrum of AHL production rates (Table 2). As AHL is constitutively produced, its buildup leads EsaR to unbind from PesoR and allow SspB production, resulting in growth arrest in minimal medium with glucose. Only the weakest two Esal expression cassettes (L25R and L31R) allowed strains to grow measurably in minimal media before leading to growth arrest (Table 1). These were transformed with the pTrc-IN01 plasmid\textsuperscript{40} to enable MI production. MI titters showed no improvement from L25R or L31R over wildtype IB1379 (Figure 7C). Pfk-1 activity profiles still showed a decline in activity in AG2349 despite the absence of Esal in this strain, indicating there was still notable leaky activity of SspB (Figure 7D).

While this system was unsuitable for controlling degradation of Pfk-1 through sspB induction, it may still be very useful in other applications where the system is not as sensitive to basal leaky expression. This may be especially true in cases where expression of a given benign pathway enzyme is controlled by PesoR, and certain levels of premature protein expression would not harm cell growth.
Figure 6. Architecture of QS-based SspB-dependent regulation of Pfk-1 activity. Expression of EsaR was driven by a constitutive promoter and binds to P_	ext{esal} in the absence of AHL. The coding sequence of Pfk-1 is appended to a SspB-dependent SsrA degradation tag (DAS+4). EsaR expression was varied by instituting a library of promoters and RBS variants from Mutalik et al. Production of AHL allowed EsaR to unbind from P_	ext{esal}, allow production of SspB which led to fast degradation of tagged Pfk-1. All components of the circuit were genomically integrated.

Figure 7. Tuning and characterization studies for the QS-based SspB-dependent system for regulation of Pfk-1. (a) IB2265 showed no response to AHL as it already had lower Pfk-1 activity in crude lysates than the IB1643 control. (b) Growth profiles in minimal medium with glucose showed that IB2265 did not grow with or without AHL, suggesting that leaky SspB levels led to low Pfk-1 activity and growth defects. After tuning down the strength of its RBS to decrease leaky production of SspB, the strain responded to AHL. AG2349 grew only in the absence of AHL. (c) Constitutive expression of EsaR was instituted by integration of promoter and RBS variants. M1 titers showed no boost over the IB1643 control. (d) Pfk-1 activity profiles during production studies showed that AG2349 (containing no EsaR expression cassette) still showed decline in activity over time. Error for (c) and (d) represented as s.d. of duplicate cultures.
2.3.2.3 Transcriptional control of gene expression through the $P_{\text{esas}}$ architecture

The transcriptional regulator, EsaRI70V, binds the $P_{\text{esas}}$ promoter in the absence of 3-oxohexanoyl-homoserine lactone (AHL)\textsuperscript{44} and activates transcription (Figure 8A). Accumulation of AHL occurs as it is produced by the AHL synthase, Esal, eventually leading to disruption of EsaRI70V binding and deactivation of the $P_{\text{esas}}$ promoter. By varying the expression level of Esal, which changed the AHL accumulation rate, we created a library of circuit variants to trigger down-regulation of the $P_{\text{esas}}$ promoter at variable times and cell densities over the course of a fermentation. Pre-characterized promoter and ribosome binding sites (RBS) from Mutalik et al\textsuperscript{31} were combinatorially assembled to scan a large expression space and achieve a spectrum of Esal expression levels (Table 2). Such tuning capabilities permit the discovery and implementation of pathway-specific optimal switching times.

This system can be used to dynamically downregulate expression of any gene of interest by placing the gene downstream of the $P_{\text{esas}}$ promoter. Degradation tags were added at the C-termini of target peptides to ensure short half-lives of transcribed proteins. The degradation tag provides an additional control mechanism for modulating protein pools and pathway fluxes in the cell. To characterize circuit behaviors and relative switching times, $esaRI70V$ was inserted in the genome of \textit{E. coli} MG1655 under a constitutive promoter from the BioFAB library (apFAB104)\textsuperscript{45}, yielding strain AG2681. \textit{esal} was subsequently integrated into a combinatorial library of promoter and RBS variants to produce a 28-strain series (named “LXX” in Table 1) with a range of AHL production rates. A $P_{\text{esas}}$ promoter driving Green Fluorescent Protein (GFP) expression on a medium copy plasmid (pCOLA-$P_{\text{esas}}$-GFP(LVA)) was then introduced into the LXX strain series (Table 1), yielding a range of rates at which GFP is switched off. Our attempts to use genomically integrated GFP driven by $P_{\text{esas}}$ resulted in inadequate fluorescence for characterization studies. However, for subsequent pathway-relevant targets in this study, all parts of the circuit, including the $P_{\text{esas}}$ promoter driving the gene of interest, were genomically integrated.

Continuous fluorescence measurements of the LXX strain series produced a variety of switching times (Figure 8B and 8C). Strain AG2681 had the highest peak fluorescence, as GFP was constitutively expressed due to the absence of Esal and AHL production; however, the inclusion of the degradation tag eventually led to a decay profile similar to that seen in those strains producing AHL as this strain neared stationary phase and naturally attenuated protein production. To compare switching rates in the various strains, the cell density corresponding to the time of maximum GFP fluorescence was determined and denoted as the “switching OD.” Generally, as the predicted strength of promoter-RBS combinations\textsuperscript{51} driving \textit{esal} increased, peak GFP fluorescence and switching OD decreased as switching occurred earlier. To confirm consistency with the predicted order of strength, strains were rank ordered based on observed switching.
times and compared with the predicted order of strength of the various Esal cassettes (Fig. 8C insets). Surprisingly, circuit switching seemed highly sensitive to Esal expression, such that most promoter-RBS combinations led to very low peak GFP and switching ODs (Fig. 8C). Only the weakest predicted combinations showed notable delays in GFP knockdown. This indicates that very low expression levels of Esal and AHL production are needed for this system to switch, even when placed in the genome.

![Diagram](image)

**Figure 8.** Characterization of a QS-circuit to dynamically modulate a target gene of interest (GOI). (a) Schematic of the engineered circuit containing a library of promoter and RBS combinations to vary Esal expression and AHL production rate, and differentially trigger downregulation of any GOI. Stronger Esal expression leads to earlier downregulation of the GOI. QS circuit components (esal, esar170V) are integrated in the genome. All promoter/RBS combinations utilized to drive esal are described in Table 2. (b) Representative fluorescence profiles for all strains containing GFP under control of the QS-circuit. Switching time and rate vary among strains containing different Esal expression cassettes. GFP is carried on a medium-copy plasmid to elicit sufficient fluorescence for detection. Fluorescence profiles for all 31 strains tested are depicted, with strain numbers indicated for clearly distinguishable profiles. (c) Switching OD for all strains containing GFP under the control of the QS circuit. Switching OD was determined by ascertaining the cell density at the point of maximum fluorescence for a given strain. Constitutive GFP expression in the absence of Esal (AG2681, green bar), basal GFP expression in the wild-type MG1655 host without the QS circuit (+ GFP, right red bar), and wildtype MG1655 alone (MG1655, left red bar) were also tested. Switching OD for all strains was plotted against predicted strength of the corresponding promoter-RBS combination driving esal, obtained from Mutalik et al.\(^{23}\) (Table 2) (left inset). All strains with corresponding Esal cassettes were also rank-ordered based on the measured switching OD (actual rank, early to late switching), and were compared to their corresponding place in the rank-ordered list of switching times based on predicted Esal strengths from Mutalik et al.\(^{23}\) (predicted rank, high to low expression) (right inset). The ranks predicted from switching OD correlated well with the previously predicted ranks, with less agreement at lower ranks (higher Esal strengths, earlier switching times). Error bars indicate s.d. of triplicate cultures.
relative trends of switching times observed through GFP characterization matched well with the previously reported order, indicating consistency and reliability in performance of our circuit and its constituent parts.

2.3.3 Dynamic gene modulation to control glycolytic flux

Given the LXX series of characterized circuit variants, we applied this module to control glycolytic flux by dynamically downregulating Pfk-1. As in earlier scenarios, zwf was deleted to prevent flux into the pentose phosphate pathway, thereby restricting G6P flux between glycolysis and MI production. All parts of the circuit were genomically integrated. The native promoter of pfkA was replaced with the Pesas promoter in the native genomic locus, and a standard SsrA degradation tag (AADENYALAA, “LAA”) was appended to the C-terminus of the protein to enable rapid removal when transcription has been halted. esaRI/70V was inserted in the genome under the control of the previously used constitutive promoter (apFAB104)45, and the final resulting strain was designated IB2275 (Table 1). This strain constitutively expressed Pfk-1 from Pesas due to the absence of Esal and AHL production. In order to allow autonomous downregulation of Pfk-1, esaI was inserted in the genome under the control of a subset of the aforementioned expression library (Tables 1 and 2). Strains were characterized in minimal medium with glucose as the sole carbon source, thereby coupling Pfk-1 expression and glycolytic flux to cell growth.

A spectrum of growth and Pfk-1 knockdown profiles was obtained (Figure 9A and 9B). With weaker Esal expression, the dynamic decay in Pfk-1 activity was slower. As seen in the GFP characterization, however, only the weakest promoter and RBS combinations allowed sufficient delay in Pfk-1 knockdown and growth arrest. Promoter-RBS combinations stronger than L24 prevented sufficient growth in minimal medium as Pfk-1 activity presumably decayed too quickly. Thus, in order to allow enough biomass to build up, and allow switching to occur at an intermediate point in the fermentation, Esal expression must be low. Switching ODs in these strains were further elucidated with plasmid-based Pesas-GFP(LVA) using the same analysis procedures as the initial circuit characterization (Figure 10). It should be noted that Pfk-1 expression under the Pesas promoter is much higher than from the wildtype promoter, as seen by the differences in activity between IB2275 (Pesas driving pfkA) and IB1379 (pfkA under its native promoter; Fig. 9C and Table 3). However, when esaI is inserted into IB2275, the Pfk-1 activity ultimately drops below the constitutive wildtype level of IB1379. Thus, the Pesas promoter has very strong “on” and “off” modes, making it suitable for dynamic pathway flux regulation. The library containing Esal variants comprises a set of strains to enable switching from “growth mode” to “production mode” at different rates in a completely autonomous fashion. The strains can be used to explore trade-offs between yield and titers, thereby allowing the point of maximum productivity to be achieved for any given target product compound branching off from central carbon metabolism.
Figure 9. QS-based valve controlling Pfk-1 expression regulates cell growth and flux into central carbon metabolism. All circuit elements are genomically integrated. (a) Growth profiles show that strains with higher Esal expression grow slower. Relative expression strengths for the strains are provided in Table 2. IB2275 does not grow as well and shows a lower final OD600. (b) Pfk-1 activity profiles in crude lysates indicate that weaker Esal expression generally trends with slower decrease in activity over time. IB2275 and IB1379 showed constitutive activity levels over all time points. Esal-containing strains displayed high Pfk-1 activities initially, but eventually decreased to below the levels in IB1379. Data are also provided in tabular format (Table 3). (c) mRNA levels from pfkA presented as a fold change from IB2275. Transcript levels corresponded to Pfk-1 activity trends. Error bars denote s.d. of triplicate cultures.

Figure 10. Circuit switching ODs, determined through continuous measurement of plasmid-based GFP, in strains containing the QS-based valve controlling Pfk-1 expression. Strains containing the genomically-integrated QS-valve controlling Pfk-1 were transformed with pCOLA-Pesal-GFP(LVA) and grown in MOPS medium with 10 g/L glucose. Switching OD was determined by ascertaining the cell density at the point of maximum fluorescence for a given strain. Constitutive GFP expression in the absence of Esal (IB2275) was also tested to determine the basal GFP degradation kinetics without active arrest in transcription through a QS circuit. Trends for these switching ODs ascertained from continuous GFP measurement match those obtained from discrete measurements of Pfk-1 activity in Fig. 9B. Error represented as s.d. of triplicate cultures.
Table 3. Pfk-1 activity values presented in Fig. 9b. Error represented as s.d. of triplicate cultures.

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2.3.4 Optimal strain for MI production

With successful autonomous control of growth using components of the Esa QS system, the strain series with variable Pfk-1 switching times was applied to improve production of commercially interesting compounds, such as MI. As described previously, MI branches from glycolysis at the G6P branchpoint and can subsequently be converted to glucaric acid (Figure 2). For MI production, strains were transformed with pTrc-INO1 (Table 1) and tested to determine if switching increases MI titers. Production tests were conducted in MOPS minimal medium supplemented with 10 g/L glucose as the sole carbon source. Not surprisingly, MI production was very poor in IB2275, given its poor growth profile and high Pfk-1 activity (Figure 11). However, MI production could be recovered back to levels equal to or above those in the wildtype IB1379 strain through EsaI expression and control of Pfk-1 activity. Pfk-1 activity profiles for the various EsaI-expressing strains (L24S, L30S, L19S, L25S, L31S) trended consistently with the initial characterization of the GFP library: stronger EsaI expression led to a faster decline in Pfk-1 activity (Figure 11A). Further, a boost in MI titers over IB1379 was obtained with an intermediate AHL production rate, as predicted by simulations (Figure 5 and 11B). Strain L19S exhibited a 20% increase in titer and yield, suggesting that it was able to balance growth and production by decreasing glycolytic flux at a more suitable switching time.
2.4 Discussion

Dynamic regulation of pathway fluxes has emerged as a new frontier in metabolic engineering\textsuperscript{35-37}. In this work, we engineered tunable genetic circuits, using parts from a bacterial QS system, to exercise autonomous, dynamic control over target metabolic genes and redirect pathway fluxes. As these circuits were built from pathway-independent parts, they could be applied in multiple pathway scenarios to regulate metabolic fluxes and achieve significant increases in titers of the corresponding products.

The majority of previous studies associated with dynamic regulation have proposed strategies to sense and respond to specific intermediates\textsuperscript{21,38}, cell states\textsuperscript{20} or medium compositions\textsuperscript{65}. While these strategies are effective and can generate interesting behaviors, such as metabolite oscillations, they are specific for the pathway or compounds for which they are developed. Control of gene expression in response to nutrient starvation provides a well-known inducer-free approach to circuit design\textsuperscript{65}. We investigated nutrient-limitation strategies to boost MI production by constructing a circuit that induces Pfk-1 degradation upon depletion of phosphate. Our findings suggested that such drastic nutrient perturbations lead to unknown global metabolic effects, implying limitations to such strategies for widespread industrial use. Use of metabolite biosensors is another strategy that has been used to dynamically control pathway gene expression, but it is limited by the need to first mine the genome to find metabolite-responsive parts\textsuperscript{20}. Our QS-based system is independent of any specific metabolites and so may be ported to any pathway with minimal labor redundancy. In the case of MI production, a flux control point – a "metabolite valve" – was installed at the G6P branchpoint to redirect flux from an endogenous pathway to heterologous product.
formation. It is important to note that while transcription of the target gene may be halted by our QS circuit, cellular pools of this protein must also be depleted to observe phenotypic changes. As protein degradation rates vary, the necessity and strength of appended degradation tags will differ on a case-by-case basis. In this work, strong degradation tags were required to adequately deplete GFP and Pfk-1 pools.

In addition to the generalizable design, our system is also tunable and fully autonomous, thereby mitigating the need for external inducer addition after inoculation. By installing a library of EsaI expression levels to endogenously produce the trigger molecule, a series of strains was created to give varying rates of switching to find the optimal one for a given pathway scenario. While dynamic pathway control, especially to switch between “growth” and “production” modes, is strongly desired by the bioprocess industry, its use has been largely limited due to incompatibility of the relevant inducers. Many inducers are physiologically active, and almost all impart prohibitively high downstream purification costs, leading industry to demand that the trigger molecules be a constituent part of the host metabolism.

As optimal production strategies become more complex, host genetic backgrounds become more cumbersome to engineer. Layered synthetic biology devices can cause severe metabolic burden on the cell, leading to suboptimal performance. The QS system presented here demonstrates a spectrum of modules with precise tunability of kinetics for adaptation to the desired pathway with minimal upfront effort and metabolic burden. In the next phase of this thesis, we sought to evaluate the robustness of this system for potential translation to industrial applications by measuring its performance in response to changes in growth medium composition and culture scales. We also measured the consistency in its performance in multi-step heterologous pathways for glucaric acid production, as well as its portability to completely new pathway and product contexts to gage its generalizability.
Chapter 3

Evaluation of robustness, scalability and extensibility to determine industrial applicability and usability
Abstract

While the QS-based transcriptional control module demonstrated advantages for myo-inositol (MI) production in minimal medium, we wanted to evaluate the context dependencies and generalizability of this device. This is especially important for future industrial applicability for synthetic biology devices in different applications, pathways, and processes. MI production trials were carried out in different medium compositions that varied between lean and defined to rich and undefined. Titer trends between different switching times were consistent across media, and the advantages of dynamic regulation became more apparent in richer medium as acetate accumulation was limited and titer boosts were larger. Furthermore, scalability analyses were carried out to verify that the titer trends seen in shake flasks were consistent in scale-up (benchtop bioreactors) and scale-down (microtiter plates) models. Extension to the complete glucaric acid pathway revealed that titer trends seen for MI production were also consistent for glucaric acid. Finally, the QS module was ported to a completely new pathway context where dynamic regulation was required. In the aromatic amino acid biosynthesis pathway, the QS module allowed dynamic downregulation of shikimate kinase, AroK, to allow accumulation of the industrially relevant compound, shikimate. This was the first demonstration of bio-accumulation of shikimate in minimal medium without any required supplementation of aromatic amino acids.

This chapter contains material adapted from:

3.1 Introduction

Synthetic biology is a highly burgeoning field with the potential to impact countless fields in science, technology, and medicine. Genetic circuits, strain engineering and microbial community building strategies are increasingly being applied to fields that were traditionally quite separate from biological and biomedical areas. Bioproduction of desired and novel compounds is emerging as one of the most prominent areas into which synthetic biology devices are being applied to make hosts more productive and efficient cell factories.

Synthetic biology devices, however, are often constructed and characterized in the laboratory with highly controlled conditions. From a process perspective, medium formulations, culture vessels and working volumes are often unchanged throughout the entire development process in the lab. Additionally, depending on the type of culture vessel used, other process parameters may not be controlled, such as pH and DO. From an applications perspective, the pathway of interest and the relevant context to which the device is applied in the lab is usually defined and unchanged. While these strict conditions may be adequate for early stage device construction, industrial applicability strongly demands a deeper characterization of the robustness of these devices to changes in process parameters and applications contexts, especially since genetic devices have been shown to have considerable variability in performance in different conditions.

The translation of a device into an industrial context may often involve a change in medium composition which can drastically change circuit performance. For example, previous studies have shown that even from simple devices that allow inducible gene expression, protein production can change by several fold when switching between different strains in *E. coli*, or the device may fail altogether. Moreover, industrial translation also requires robustness to changes in culture scales and configurations. For example, process development and optimization is often carried out using both scale-up and scale-down culture models. Microplates, shake flasks, and stirred tank bioreactors are all used to maximize product formation. Industrial production often requires large culture vessels, which may suffer from incomplete mixing and fluid dynamics than those in smaller lab-scale vessels. As such, it is vital for genetic devices to be robust to changes in these scales. This is especially relevant for quorum sensing (QS)-based devices for which local gradients of AHL may preclude synchronized behavior of the culture, such as synchronized dynamic switching of pathway gene expression.

In addition to changes in process conditions, variations in the desired application in which the genetic device is applied are also common. This is especially relevant for devices that are proposed to be "generalizable" and "pathway-independent". The QS-based transcriptional control module proposed in this thesis can
theoretically be applied to any pathway of interest to downregulate any target gene. This level of portability increases its applicability in industry. Previous reports, however, have proposed that genetic devices can be highly context dependent and can fail when shifted to new applications\textsuperscript{71}. As such, it is vital to demonstrate that our QS device is modular and easily portable to completely new contexts, beyond MI production, where dynamic regulation may be desired.

With this in mind, we explored the robustness of our QS-based dynamic gene regulation device in the face of changes in growth medium composition, culture scales and configurations. Results revealed that the device was functional across all medium compositions tested, and dynamic strains showed an increasing advantage in titers over strains with no dynamic pathway regulation. Using dynamic pathway control, MI titers were increased 5.5x and glucaric acid titers were increased from unmeasurable to >0.8 g/L in a rich, undefined, industrially relevant medium composition. Consistency of device performance was verified in benchtop bioreactors, achieving nearly 10-fold and 5-fold boosts in specific titers of MI and glucaric acid, respectively. In addition to robustness to changes in process parameters, we also sought to demonstrate the portability of our device to completely new pathway contexts. The control module was applied to dynamic downregulation of flux into aromatic amino acid biosynthesis to accumulate the industrially-relevant intermediate, shikimate, resulting in an increase in titers from unmeasurable quantities to >100 mg/L.

3.2 Material and Methods

All strains and plasmids used in this study are summarized in Table 4 and 1, respectively.

3.2.1 Strain construction

3.2.1.1 Preparing strains for glucaric acid production

To prepare strains to make glucaric acid, the $\lambda$DE3 lysogen was first inserted into strains L24S, L19S, L31S and IB1379 using a $\lambda$DE3 Lysogenization Kit (Novagen, Darmstadt, Germany). Next, in order to eliminate catabolism of glucaric acid and the pathway intermediate glucuronic acid, uxaC and guaD were sequentially deleted from these strains via P1 transduction from the Keio collection donor strains\textsuperscript{72}, yielding strains L24SGA, L19SGA, L31SGA and IB1379GA (Table 4).

3.2.1.2 QS-based transcriptional control of AroK

For QS-based dynamic control of the shikimate pathway, aroK was ligated to the $P_{spe}$ promoter, and this combined piece was ligated to an expression cassette consisting of esaRI70V driven by a synthetic promoter (apFAB104). A weak SsrA tag (AANDENYAASV, “ASV”) was appended to the C-terminus of aroK,
yielding \textit{esaRI70V-peso5-aroK(ASV)}. This cassette was genomically integrated, using "clonetegration"\cite{47}, into the HK022 locus of a MG1655 strain in which the native copies of \textit{aroK} and \textit{aroL} were deleted, yielding strain AG2310. The Esal expression library was installed as described in chapter 2.

3.2.2 Culturing and fermentations

3.2.2.1 Shake flask fermentations

Shake flask fermentations to measure MI production were carried out in MOPS medium or MOPS + 0.2% casamino acids, as described in chapter 2. Fermentations were also carried out in T12 medium containing 10 g/L D-glucose, 7.5 g/L yeast extract, 7.5 g/L soy peptone, 7 g/L Na$_2$HPO$_4$, 3 g/L KH$_2$PO$_4$, 0.5 g/L NaCl, 3 g/L (NH$_4$)$_2$SO$_4$, 4 mM MgSO$_4$, as well as 100 μg/ml carbenicillin for plasmid maintenance. Overnight 3-ml cultures were started in T12 medium at 30 °C, and then diluted 1:100 into fresh 30-ml starters to grow for ~5 hours until exponential phase was achieved. 60-ml working cultures, supplemented with 50 μM IPTG, were then inoculated at OD$_{600}$ 0.05. Flasks were incubated at 30°C with 250 rpm shaking and 80% humidity. Samples were taken periodically for measurement of enzyme activity and extracellular metabolites. Fermentations were carried out for 64 hrs, at which point all glucose was consumed.

Glucaric acid production trials were conducted in T12 medium containing 10 g/L glucose as described above. The \textit{λDE3} lysogen was inserted into the strains to be tested (noted above), and the resultant strains were transformed with pRSFD-IN-MI and pTrc-Udh (Table 1). The medium was supplemented with 100 μg/ml carbenicillin and 50 μg/ml kanamycin for plasmid maintenance. Overnight cultures and reinoculation were carried out as described above for MI production. 60-ml working cultures, supplemented with 100 μM IPTG to induce the glucaric acid pathway enzymes, were inoculated at OD$_{600}$ 0.05, and flasks were incubated at 30°C with 250 rpm shaking and 80% humidity. Samples were taken periodically to measure extracellular metabolites.

Shikimate production trials were conducted in modified MOPS medium containing 10g/L glucose as described above. Strains were initially grown overnight in 3-ml LB medium and then diluted 1:100 into 5-ml seed cultures of modified MOPS medium for ~24 hrs at 30 °C. These were used to inoculate 50-ml working flask cultures at OD 0.05. Flasks were incubated at 30°C with 250 rpm shaking and 80% humidity. Samples were taken periodically for measurement of extracellular metabolites, and fermentations were carried out for 72 hrs until all glucose was consumed.

3.2.3 Production trials in bioreactors
Scale-up production trials for MI were carried out in either the modified MOPS minimal medium described previously or T12 medium in a standard 3L benchtop bioreactor (Infors HT, Bottmingen, Switzerland). For MOPS trials, overnight LB cultures were used to inoculate seed cultures in modified MOPS minimal medium, then grown at 30 °C for ~24 hrs. For trials in T12 medium, cultures were grown overnight in T12 medium at 30 °C. These were diluted 1:100 in fresh T12 medium and allowed to grow for ~5 hrs until exponential phase was achieved. In both cases, a 1.25 L working culture, containing 50 µM IPTG to induce INO1, was inoculated to an initial OD600 0.05 for MI production. All cultivations were done in the presence of 100 µg/ml carbenicillin for plasmid maintenance. During fermentation, oxygen was supplied by filtered air at 0.75 liters/min and agitation was adjusted to maintain a 35% dissolved oxygen level (250 - 750 rpm). The pH of the culture was set to 7.0 and controlled by addition of 4 M NaOH or 4 M HCl. The temperature of the culture was maintained at 30 °C. Samples were taken periodically for measurement of extracellular metabolites and dry cell weight (calculated from measured OD600 values as 0.47*OD600). Two independent trials were run for each bioreactor experiment.

Glucaric acid production trials in T12 medium were carried out with a similar protocol as MI production trials with the following changes. For maintenance of both plasmids, 100 µg/ml carbenicillin and 50 µg/ml kanamycin were used. Induction of all pathway enzymes was achieved with 100 µM IPTG.

3.2.4 Phosphofructokinase activity assays

Samples for activity assays of Pfk-1 were collected over the course of the fermentations. Activity measurements were carried out according to the protocol reported previously25 and in chapter 2.

3.2.5 Quantification of extracellular metabolites

Glucose, MI and acetate levels were quantified by high performance liquid chromatography (HPLC) as described in chapter 2. For glucaric acid, column temperature was set to 65 °C and refractive index detector temperature was set to 35 °C.

Shikimate was quantified with a ZORBAX SB-Aq column (4.6 mm x 150 mm x 3.5 µm). The HPLC was run with a mixture of solution A (20 % methanol) and solution B (25 mM Ammonium formate, pH 2.5) as eluant at a flowrate of 0.6 ml/min. The following gradient was used: at 0-2 min, 2% solution A and 98% solution B; by 17 min, 10 % solution A and 90% solution B; by 18 min, 100% solution A and 0% solution B; at 18-19 min, 100% solution A and 0% solution B; by 20 min, 2% solution A and 98% solution B; at 20-22 min, 2% solution A and 98% solution B. Compounds were quantified with 10 µl injections using diode-array detection.
Table 4: New strains and their genotypes used in chapter 3. Data presented in chapter 3 also includes strains introduced in chapter 2 (Table 1).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB1379GA</td>
<td>MG1655(DE3) ΔendA Δzwf ΔpfkB ΔuxaC ΔgudD</td>
<td>IB1379 + λDE3 lysogen + uxaC and gudD knockout</td>
</tr>
<tr>
<td>L24GA</td>
<td>MG1655(DE3) ΔendA Δzwf ΔpfkB ΔuxaC ΔgudD</td>
<td>L24S + λDE3 lysogen + uxaC and gudD knockout</td>
</tr>
<tr>
<td>L19GA</td>
<td>MG1655(DE3) ΔendA Δzwf ΔpfkB ΔuxaC ΔgudD</td>
<td>See description for L24SGA</td>
</tr>
<tr>
<td>L31GA</td>
<td>MG1655(DE3) ΔendA Δzwf ΔpfkB ΔuxaC ΔgudD</td>
<td>See description for L24SGA</td>
</tr>
<tr>
<td>AG2310</td>
<td>MG1655 ΔaroK ΔaroL HK022::esaR70V-P_{esa}-aroK(ASV)</td>
<td>Native shikimate kinases deleted and aroK under the control of the Pesas promoter</td>
</tr>
<tr>
<td>L24SA</td>
<td>MG1655 ΔaroK ΔaroL HK022::esaR70V-P_{esa}-aroK(ASV) 186(O)::apFAB295-apFAB699-esal</td>
<td>AG + esaI driven by selected promoter and RBS sequences from Mutalik et al; Predicted strength: 4.8%</td>
</tr>
<tr>
<td>L19SA</td>
<td>MG1655 ΔaroK ΔaroL HK022::esaR70V-P_{esa}-aroK(ASV) 186(O)::apFAB296-apFAB700-esal</td>
<td>See description for L24SA; Predicted strength: 1.7%</td>
</tr>
<tr>
<td>L25SA</td>
<td>MG1655 ΔaroK ΔaroL HK022::esaR70V-P_{esa}-aroK(ASV) 186(O)::apFAB295-apFAB700-esal</td>
<td>See description for L24SA; Predicted strength: 1.3%</td>
</tr>
<tr>
<td>L31SA</td>
<td>MG1655 ΔaroK ΔaroL HK022::esaR70V-P_{esa}-aroK(ASV) 186(O)::apFAB65-apFAB700-esal</td>
<td>See description for L24SA; Predicted strength: 1.2%</td>
</tr>
</tbody>
</table>

3.3 Results

3.3.1 Evaluation of QS-based dynamic regulation device in different growth media

Industrial production contexts can vary greatly, depending on the pathway and production process. Medium compositions are often optimized with a variety of considerations, including cellular physiology, enzyme
expression and even process economics. As such, exact recipes can vary greatly across bioproduction applications and may include defined and undefined components.

In chapter 2, all production experiments were performed in lean, defined MOPS-based medium. While MOPS-based minimal media are commonly used in physiological and characterization studies, scale-up towards industrially-relevant production will require media formulations tailored to the target pathway and engineered strain. Since previous studies have shown that genetic devices can fail as a function of medium choice\textsuperscript{14}, we explored the utility of our device in two additional media in a subset of strains. In the first case, MOPS-based minimal medium containing 10 g/L glucose was further supplemented with 0.2% casamino acids. Pfk-1 activity profiles again showed stronger Esal expression leading to faster activity decline and growth arrest (Figure 12A). MI titers again showed that L19S performed best and led to an 83% improvement over IB1379 (Figure 12C).

We next evaluated strain performance in T12 medium, a phosphate-buffered formulation supplemented with yeast extract and soy peptone, that has been developed for industrial bioproduction of glucaric acid\textsuperscript{41}. T12 allowed faster growth and consumption of glucose, suggesting that it may allow further increases in productivity for a given batch-time compared to MOPS minimal medium. In this medium, a significant production of acetate was observed in strains where Pfk-1 was either constitutively expressed (IB2275 and IB1379) or suppressed too late (L31S) (Figure 12B and 12D). Despite the buffering capacity of the medium, the production of excessive acetate due to overflow metabolism led to a decrease in culture pH (pH~5.5) and premature growth arrest. Surprisingly, this was also true for the wildtype IB1379 in which basal Pfk-1 activity is considerably lower than in the QS-based IB2275, indicating that even the minimal wildtype Pfk-1 levels are severely suboptimal in constitutive mode. On the other hand, strains L19S and L24S were able to suppress Pfk-1 activity earlier, resulting in no acetate surplus by the end of the fermentation and maintenance of neutral pH. These strains had a significantly higher MI production, with L24S and L19S yielding ~3.6-fold and ~5.5-fold boosts, respectively, in endpoint MI titers over IB1379 (Figure 12D). Thus, a dynamic strategy was critical to obtain notable titers of MI in a rich formulation.
3.3.2 Application of dynamic flux regulation for glucaric acid production

While MI titer is an important indicator of circuit performance at the G6P branchpoint, the final product of the pathway that we were interested in was glucaric acid. Glucaric acid production in the presence of dynamic flux control was evaluated, which requires two further enzymatic steps downstream of MI (Figure 13A). The first is mediated by myo-inositol oxygenase (MIOX) to produce glucuronic acid, followed by uronate dehydrogenase (Udh) to make glucaric acid\(^\text{\textsuperscript{10}}\). Relevant strains (L24SGA, L19SGA, L31SGA and
IB1379GA) were prepared from L24S, L19S, L31S and IB1379 by inserting the λDE3 lysogen to allow T7-based expression of pathway enzymes, and deleting the uxaC and gudD genes to prevent consumption of glucaric acid or the intermediate, glucuronic acid (Table 4). Glucaric acid production from glucose has been poor in wild-type K strains, with titers <0.1 g/L.$^{41,71}$ Fermentations were carried out in T12 medium which has been developed for industrial bioproduction of glucaric acid.$^{41}$ The IB1379GA control strain with Pfk-1 under the wildtype constitutive promoter showed no detectable production of glucaric acid and instead accumulated acetate (Figure 13B). On the other hand, our best engineered strain, L19GA, produced titers up to ~0.85 g/L with minimal acetate accumulation. Similar to MI production in T12 medium, when Pfk-1 activity was not suppressed early enough or was constitutive (L31GA and IB1379GA), acetate accumulation and low culture pH was observed. In this case, the effect on product titers was even more drastic than during MI production (Figure 13B), suggesting that dynamic control is vital.

**Figure 13.** Glucaric acid production using the QS valve at the G6P branchpoint. (a) Schematic of the glucaric acid production pathway from the G6P branchpoint. MI is converted to glucaric acid by MOX, which is then converted to glucaric acid by Udh. uxaC and gudD encode native E. coli enzymes that metabolize glucuronic and glucaric acid, respectively, and were deleted to prevent unwanted metabolite consumption. The first step of the pentose phosphate pathway (PPP), zwf, was also deleted. (b) Glucaric acid and acetate production in strains with varying Esal expression cassettes. The best producer of glucaric acid is L19GA, which had previously shown the best MI production. With slower (L31GA) or no (IB1379GA) Pfk-1 downregulation, high acetate but no glucaric acid was detected, as seen previously with MI production in T12 medium. Error bars denote s.d. of triplicate cultures.

### 3.3.3 Scalability analysis to determine robustness and consistency of device performance

Although small-scale shake flask cultures were promising, evaluation of our QS-based strategy in more industrially relevant process development conditions, such as in a stirred-tank bioreactor, is important to justify the potential of this approach for commercial applications.$^{14}$ Bioreactors allow for more consistent
culture conditions due to improved mixing and implementation of environmental setpoints such as dissolved oxygen (DO) and pH. In addition, scale-down models of bioproduction processes are becoming increasingly important in industry, as researchers attempt to use high-throughput methods to construct and screen a large number of conditions for product formation. Therefore, while industrial application requires functionality in bioreactors, it also is increasingly important that the synthetic biology device be functional at scales smaller than typical shake flask cultures, such as microtiter plates. All data shown so far has been collected in 250-ml shake flasks with working volumes of 50-60 ml. In this section, we evaluated MI and glucaric acid production in 3L benchtop bioreactors with working volumes of 1.25L and microtiter plates with 1-mL culture volumes.

Preliminary trials of MI production in MOPS medium showed the performance in a bench-top bioreactor to be consistent with the initial shake flask results, with a 1.2-fold to 2-fold increase in titer and yield with L19S, compared to IB1379 (Figure 14). We also evaluated performance in T12 medium. With implementation of pH control, we hypothesized that despite high acetate production in IB1379, premature growth arrest in T12 medium could be avoided by controlling culture pH. IB1379 did reach a much higher final OD₆₀₀ than L19S (Table 5), and there was no residual acetate at the end of the fermentation, presumably because it was reassimilated by the cells. By comparison, L19S reached a lower OD₆₀₀ but yielded an almost 10-fold increase in specific MI titer. When we ran MI production trials in microplates in the Biolector microbioreactor system, however, we did not get similar trends as in shake flasks. None of the strains produced any acetate, for all strains tested (Figure 15A). It is unclear why these trends appeared in microtiter plates, since both shake flasks and bioreactors showed similar results. One hypothesis is that acetate production may have been mitigated because microtiter plates may have less oxygen limitation that the other two scales, leading to more balance between fluxes in glycolysis and the downstream respiration reactions and therefore, less acetate production.

We also evaluated glucaric acid production from IB1379GA and L19GA in T12 medium in bioreactors. As seen with MI production trials in bioreactors, there was no residual acetate in IB1379GA fermentations, biomass accumulation was higher than L19GA, and product titers were low (Table 5). L19GA produced a nearly 4-fold increase in titers over IB1379GA by producing almost 0.7 g/L of glucaric acid (Table 5). In microtiter plates with 1-ml cultures, we achieved the same trends as in shake flask fermentations for glucaric acid production. Namely, static strains, such as IB1379GA, or strains that switch very late, such as L31GA, produce acetate and no glucaric acid. On the other hand, L19GA accumulates no acetate and produces >0.8 g/L glucaric acid (Figure 15B).
Figure 14. MI production in a 3L bioreactor in MOPS minimal medium supplemented with 10 g/L glucose. IB1379 reached a higher final OD600 than L19S but had more than 2-fold lower specific titer.

Table 5. MI and glucaric acid production in T12 medium in a 3L bioreactor. QS-based dynamic control of Pfk-1 activity boosted specific titers of MI by almost 10-fold and of glucaric acid by almost 5-fold, indicating that the QS device functions consistently across shake flasks and bioreactor scales. With QS-based Pfk-1 downregulation, cultures reached a lower biomass and gave higher product titers, presumably due to the redirection of flux from central carbon metabolism into heterologous production. DCW refers to the endpoint dry cell weight in the culture. Error represented as s.d. of duplicate bioreactor runs.
3.3.4 Application of device to new pathway contexts: Shikimate accumulation

In order to test the utility of the Esa QS device in other pathways, we installed this device at a completely different metabolic branchpoint in which dynamic flux regulation is required to improve titers. Shikimate is an intermediate in the chorismate pathway which eventually leads to production of aromatic amino acids (AAA)\(^7\) (Figure 16A). It is also a biologically important compound that is an essential starting material for the synthesis of neuraminidase inhibitors, such as Tamiflu\(^5\), effective in the treatment of influenza\(^7\). Attempts at bioproduction of shikimate have traditionally involved deleting the genes for shikimate kinases, \(aroK\) and \(aroL\), such that shikimate cannot be converted to shikimate-3-phosphate\(^7\) \(4\). The fermentation medium must consequently be supplemented with expensive AAA to facilitate growth\(^4\). Recent academic studies and patent literature have suggested that dynamically downregulating AroK and AroL can mitigate the need for such supplementation, as AAA can be synthesized in the first phase of the fermentation to allow growth, followed by shikimate accumulation\(^7\) \(74\). Unlike the G6P branchpoint where endogenous flux was siphoned into a heterologous pathway for glucaric acid production, this case requires the accumulation of an endogenous metabolite and thereby presents an alternative scenario for the relevance of autonomous, dynamic pathway modulation.

To test our hypothesis, we used our device to dynamically control AroK expression. \(aroL\) was deleted, while a weak SsrA degradation tag (AANDENYAASV, “ASV”) was added to \(aroK\) and placed under the \(Pas\) promoter in the wildtype MG1655 genome. \(esaRI/70V\) was also driven from a constitutive promoter (apFAB104)\(^4\) in the genome, resulting in strain AG2310 (Table 4). It is important to note that initial attempts to add stronger SsrA degradation tags to genomic \(aroK\) prevented the strain from growing in MOPS minimal medium without AAA supplementation, again demonstrating the sensitivity of cell viability to changes in endogenous gene expression. In order to implement dynamic downregulation of AroK,
selected weak cassettes from the Esal expression library were inserted in strain AG2310 leading to a set of strains with different switching times (L24SA, L19SA, L25SA, and L31SA; Table 4). Cultivation in MOPS minimal medium resulted in varied shikimate accumulation, with an intermediate switching time yielding the highest shikimate accumulation of more than 105 mg/L (Figure 16B). Strain AG2310 and wildtype MG1655 were able to grow in minimal medium without AAA supplementation but were unable to accumulate any shikimate with constitutive AroK expression. To our knowledge, this is the first demonstration of shikimate accumulation in defined, minimal medium without addition of AAA, validating our Esa QS device as a generalizable tool for dynamic pathway modulation.

Figure 16. Shikimate production through the aromatic amino acid (AAA) biosynthesis pathway using the QS valve. (a) Shikimate is an intermediate in the AAA biosynthesis pathway, whose accumulation competes with production of the AAA. To enable accumulation of shikimate without supplementing with costly AAA, AroK was dynamically downregulated using the QS valve. Native copies of aroK and aroL, the shikimate kinase isozymes, were deleted. (b) Shikimate titers in strains with various Esal expression cassettes. With constitutive AroK expression, as in wildtype MG1655 or in a QS strain lacking Esal (AG2310), there is no accumulation of shikimate. With differential Esal expression, an optimal strain emerges that maximizes the production of shikimate to >100 mg/L. Error bars denote s.d. of triplicate cultures.
3.4 Discussion

In this chapter, we sought to vary the production context into which the dynamic regulation control module was applied to evaluate how its performance varies. Such evaluations are necessary to determine industrial applicability of such a module, as previous literature has stressed the context-dependence of numerous synthetic biology devices. When applied to production of MI in different medium compositions that varied from lean to rich, the dynamic regulation module actuating downregulation of \( \text{Pfk-1} \) was able to consistently yield improvements in MI titers over strains that did not contain any dynamic downregulation of \( \text{Pfk-1} \). As we moved to richer medium compositions, the advantage in MI titers achieved by dynamic strains over static strains also increased. Acetate production was also curbed in dynamic strains, preventing premature cell death due to excess acidity in shake flasks as observed with static strains. Similar trends were also observed for glucaric acid production trials in rich medium, indicating that the advantage conferred by dynamic downregulation of \( \text{Pfk-1} \) on MI titers carries over through subsequent pathway steps to glucaric acid production.

In addition to evaluations in different medium compositions, a scalability analysis was also performed at three different culture configurations and volumes—1-ml microtiter plates, 250-ml shake flasks and a 3L bioreactor. The dynamic regulation module was functional at all scales and was able to show similar improvements in product titers. In bioreactors, acetate production in IB1379 and IB1379GA did not lead to a drop in pH due to external pH control, and the acetate was reassimilated to be converted into biomass. On the other hand, L19S and L19GA had lower biomass and higher product titers than their static counterparts. Such evaluations in different scales confirmed our understanding of how carbon flux was being modulated by the QS device at the G6P branchpoint. It was also reassuring to know that despite any variations in culture contexts that occur at different configurations, the QS device performed similarly, indicating it is robust to changes in culture scales.

One of the major advantages of a QS-based dynamic regulation device is that it is not dependent on any intermediates of any potential production pathway. Therefore, it is "generalizable" and could be applied to dynamically regulate expression of any target gene. In order to demonstrate this portability, we applied our device to dynamically downregulate expression of the shikimate kinase, AroK, and thereby allow cells to accumulate shikimate. To our knowledge, this was the first demonstration of shikimate accumulation in biological cells in minimal medium (with glucose as the sole carbon source) without any supplementation of the expensive aromatic amino acids. While our shikimate titers were much lower than the best shikimate producers in literature, we simply sought to demonstrate the portability and functionality of our device in
new pathway contexts. Therefore, no process or strain optimization was performed to necessarily boost shikimate titers.

Through our various types of device characterization experiments, we sought to propose an example workflow for development of synthetic biology devices. While actual translation to industry would certainly require more extensive studies to quantify the robustness and context dependencies of the devices, all of which would require extensive capital investment, academic developers can advance their own designs towards industry by carrying out such basic characterization studies. Performance evaluations of genetic modules to slight perturbations, such as those carried out in this thesis, can prompt further investigations and encourage researchers to work with an application-oriented mindset. As bioproduction becomes an increasingly common strategy to produce desired compounds, the integration of synthetic biology modules for strain engineering is gaining traction. With such wide demand and potential applicability, it is paramount that academic researchers encourage collaboration and translation of new technologies to the bioproduction industry.
Chapter 4

Building complexity to allow higher order autonomous control of metabolic pathways
Abstract

Dynamic regulation at given metabolic points in a pathway may be necessitated by multiple reasons. In the glucaric acid pathway, redirection of glycolytic flux for product formation has been shown to improve final titers. In addition, delayed induction of myo-inositol oxygenase (MIOX) at intermediate points after inoculation has been shown to improve endpoint pathway productivity and titers. A myo-inositol (MI) biosensor developed in the Prather lab allows induction of MIOX upon buildup of MI pools at intermediate points in the fermentation. Switching times can be varied by modulating expression of the cognate transcription factor in the circuit. Using this strategy independently boosted glucaric acid titers by 2-fold. Upon combining the QS-based downregulation of Pfk-1 and MI-biosensor based induction of MIOX in one strain, two dynamic gene regulation devices instituted at different metabolic points were layered together. Each device was independently tunable with respect to switching time, leading a two-dimensional combination space in which each device could be triggered at different times. All constructed combinations were rigorously evaluated for glucaric acid production. Titers revealed synergistic boosts in endpoint titers and the best combination of switching times resulted in the highest glucaric acid titer achieved to date, ~2 g/L. Scaleup in benchtop bioreactors showed that performance was consistent with shake flasks.

This chapter contains work and data collected in conjunction with Stephanie Doong from the Prather lab. It is adapted from a manuscript in preparation.
4.1 Introduction

The QS-based dynamic regulation strategy developed so far offers one way to upregulate or downregulate gene expression over the course of the fermentation. In each scenario, however, a single strategy was implemented to modulate gene expression in a given strain. In the case of glucaric acid production and shikimate production, QS-based dynamic downregulation of a single gene led to notable improvements in product titers. Conceivably, however, multiple control points may exist in a given pathway where implementation of dynamic gene regulation might boost pathway performance.

In the case of the glucaric acid pathway, the Prather lab has discovered that delaying the expression of the myo-inositol oxygenase (MIOX) enzyme, which converts myo-inositol (MI) into glucuronic acid, can notably improve glucaric acid titers\(^{33}\). It is hypothesized that the presence of MI can improve MIOX stability and solubility, thereby promoting increased production of glucuronic and glucaric acid from the pathway. Delaying expression of MIOX until an intermediate point in the fermentation can allow intracellular MI pools to build up and enhance MIOX activity. Experimental results with inducible MIOX expression have revealed that triggering MIOX expression up to 10 hours post-inoculation (late exponential/early stationary phase) can improve endpoint pathway titers compared to when MIOX is induced at inoculum with the rest of the pathway\(^{33}\). These results suggest an additional opportunity to improve glucaric acid pathway output through dynamic regulation, beyond restricting glycolytic flux. As redirecting glycolytic flux and enhancing MIOX performance represent two independent control points to improve glucaric acid titers, we hypothesized that implementing dynamic control strategies at both points simultaneously may provide synergistic improvements in titers beyond that provided by each strategy independently. This, however, would require additional modules for dynamic gene regulation that would be orthogonal to our QS-based transcriptional control module, but could act in parallel with it.

Ongoing work in the Prather lab has led to the development of a MI biosensor circuit. This circuit can be used to induce expression of a target gene in response to the presence of MI. The parts for this system, including the MI-responsive transcription factor and the sequence of its cognate DNA binding site, have been imported from \textit{Corynebacterium glutamicum} into \textit{E. coli}. Specifically, the transcription factor, IpsA, binds to DNA at its cognate sequence in the absence of MI. The cognate sequence was inserted between the -35 and -10 consensus sequences of standard, well-characterized \textit{E. coli} promoters to create a "hybrid" promoter (Phybna) whose activity would be responsive to IpsA binding (Figure 17A). In the absence of MI, IpsA would be bound to the promoter and repress transcription. External addition or endogenous production of MI and its interaction with IpsA would cause IpsA to be released from the DNA and allow gene expression to proceed (Figure 17B). This biosensor was used to drive expression of MIOX, such that its
expression would only be activated once MI pools have accumulated sometime into the fermentation. When this biosensor circuit was used to delay expression of MIOX in production trials, we achieved more than a 2-fold improvement in glucaric acid titers compared to strains in which MIOX was induced at inoculum.

After evaluating the benefit of this strategy on its own, this circuit was then imported into strains that already contained the QS-based module to downregulate Pfk-1, thereby instituting two layers of independent dynamic control at different points in metabolism. These composite strains were able to achieve a 10-fold boost in glucaric acid titers over strains that contained no dynamic regulation at all. This is the highest glucaric acid titer reported in *E. coli* K strains to date. Scale-up to bioreactors showed that this synergistic boost in titers was remarkably consistent. This system demonstrates an example of coupling pathway-dependent (MI-inducible IpsA circuit) and pathway-independent (QS-based transcriptional control circuit) strategies in the same strain for maximizing performance towards desired objectives.

With two orthogonal strategies dynamically regulating different genes in metabolism, we are moving closer to a “just-in-time transcription” strategy, where pathway genes are only expressed when needed. Such designs already exist for many endogenous pathways and require many levels of native dynamic regulation to modulate multiple genes\(^5\). We envision similar levels of control for heterologous pathways to maximize titers.

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**Figure 17. Construction and characterization of the MI biosensor.** (a) Architecture of the hybrid promoter. (b) Biosensor circuit, where a constitutive promoter expressing IpsA represses the hybrid promoter driving expression of the gene of interest (GOI). Presence of MI prevents IpsA-mediated promoter repression.
4.2 Materials and Methods

All plasmids used in this study are summarized in Table 6.

4.2.1 Construction of the Myo-inositol Biosensor

The MI-responsive hybrid promoter (P_{hybrid}) was constructed by placing the IpsA binding site sequence between the -35 and -10 consensus sequences of an E. coli promoter, J23101, obtained from the Anderson promoter library. The sequence of P_{hybrid} and the important sites are shown below. The -35 and -10 consensus sequences are underlined, the IpsA binding site is denoted in blue, and the putative transcription start site is shown in red:

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ttgac tttattgattcagttattatgctagca
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The hybrid promoter (P_{hybrid}), GFP with terminator, and the plasmid pHHD01K {superscript 78} were assembled to form pHH-P_{hybrid}-GFP. IpsA was amplified from C. glutamicum genomic DNA and recognition sites for EcoR1 and BamHI were appended on either end. The PCR product was digested and ligated into pHH-P_{hybrid}-GFP to construct pHH-P_{hybrid}-GFP-P_{let}-ipsA. To create the set of MI biosensor plasmids with varying constitutive IpsA levels, the P_{let} promoter was replaced with promoters from the Anderson promoter library via digestion with XhoI and KpnI and ligation. The sequence of the different promoters used to drive IpsA are given below, along with their denotations in the Anderson promoter library as well as their corresponding names in this thesis. Capital letters represent the variable regions of the promoter through which promoter strength was varied:

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Anderson Promoter Library part ID</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>P1</td>
<td>J23111</td>
<td>TTGACGgctagctagctagttagTATAGTgtagca</td>
</tr>
<tr>
<td>P2</td>
<td>J23106</td>
<td>TTTACCGgctagctagctagttagTATAGTgtagca</td>
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<td>J23104</td>
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<td>P5</td>
<td>J23107</td>
<td>TTTACCGgctagctagctagccctaggtTATTATgtagc</td>
</tr>
</tbody>
</table>

For dynamic control of MIOX, GFP was replaced with MIOX in the various biosensor plasmids.

For glucaric acid production under control of the MI biosensor, small tweaks to the original pathway plasmids {superscript 40} were made. The plasmid pRSFDCarb-IN-Udh, encoding enzymes Ino1 and Udh, is a modified form of the duet vector pRSFD-IN-Udh, where the kanamycin resistance was swapped for carbenicillin resistance. MIOX was contained on the biosensor plasmids described above.
4.2.1.1 Measuring Anderson promoter strength

We utilized a few different promoter variants from the Anderson promoter library to drive IpsA expression and thereby obtain different levels of IpsA expression. Each promoter was used to derive RFP expression and endpoint fluorescence was measured.

4.2.2 Measuring GFP with exogenously added and internally produced MI

4.2.2.1 Exogenously added MI

Biosensor plasmids containing GFP driven by P\text{hybrid} were transformed into MG1655(DE3) and grown in LB medium with 50 \( \mu \)g/ml kanamycin. Overnight cultures were used to inoculate fresh 1-ml LB+kanamycin cultures in 48-well flower plates for incubation in the Biolector microbioreactor system. Appropriate concentrations of MI were added as needed for each individual sample to induce GFP production. Cultures were incubated at 30 °C, 1200 RPM shaking and 80% humidity. GFP fluorescence (Ex/Em: 488/520 nm) and biomass were measured continuously for 24 hrs.

4.2.2.2 Intracellularly produced MI

Biosensor plasmids containing GFP driven by P\text{hybrid} and plasmid pTrc-INO1 (Table 1) were co-transformed into MG1655 (DE3) cells. Cultures were grown in T12 medium (10 g/L D-glucose, 7.5 g/L yeast extract, 7.5 g/L soy peptone, 7 g/L Na\text{2}HPO\text{4}, 3 g/L KH\text{2}PO\text{4}, 0.5 g/L NaCl, 3 g/L (NH\text{4})\text{2}SO\text{4}, 4 mM MgSO\text{4}) plus 50 \( \mu \)g/mL kanamycin and 100 \( \mu \)g/mL carbenicillin. Fermentation was carried out for 48 hours in the BioLector (30°C, 1200 rpm, 80% humidity). INO1 expression was induced with 50 \( \mu \)M isopropyl \( \beta \)-D-1-thiogalactopyranoside (IPTG) at inoculation. Biomass and GFP were monitored as described above. For endpoint MI titers, 100 \( \mu \)L culture from each well was removed, centrifuged at 3000 × g, and the supernatant retained for titer measurements.

4.2.3 Glucaric acid Production

Biosensor plasmids containing MIOX driven by P\text{hybrid} and pRSFDCarb-IN-Udh were transformed into strains MG1655 (DE3) from which guaD and uxaC were deleted. Overnight cultures in T12 medium were used to inoculate 30 mL starter cultures at approximately at a dilution of 1:50. Starter cultures were incubated for 4-8 hours until mid-exponential phase was achieved, and used to inoculate 50 mL fermentation cultures at OD 0.05. Fermentation cultures were incubated for 72 hours at 30°C, 250 rpm and 80% humidity. INO1 and Udh expression was induced with 100 \( \mu \)M isopropyl \( \beta \)-D-1-thiogalactopyranoside (IPTG) at inoculation. Samples were taken every 24 hours for OD and titer measurements.
Production trials were also carried out in previously constructed strains containing QS-based dynamic downregulation of Pfk-1\(^7\). Specifically, biosensor plasmids driving MIOX expression from \(P_{\text{hybrid}}\) as well as pRSFDCarb-IN-Udh were co-transformed into IB1379GA, L24GA, L19GA, and L31GA strains in a combinatorial fashion, such that we had every combination of switching times for both Pfk-1 knockdown and MIOX induction. This led to 24 different combinations (4 QS strains x 4 biosensor plasmids, plus 4 no-plasmid controls). Fermentation trials were conducted in these strains in T12 medium as described above. OD and titer measurements were taken.

### 4.2.4 Production trials in bioreactors

Glucaric acid production trials in bioreactors were carried out for a subset of strains that contained dual dynamic regulation (Pfk-1 downregulation and delayed MIOX induction). Fermentations were carried out in T12 medium using the same procedure as described in chapter 3 for glucaric acid production in bioreactors.

### 4.2.5 Measurement of extracellular metabolites

Metabolites were quantified using HPLC using the same protocol described in chapter 2 and 3.

**Table 6. Plasmids used in this study**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHHD01K</td>
<td>Empty vector, parent plasmid for biosensors</td>
<td>78</td>
</tr>
<tr>
<td>pHH-P(_{\text{hybrid}})-GFP-P1-ipsA</td>
<td>pHHD01K with GFP expressed from hybrid promoter; IpsA expressed from constitutive promoters P1-P5</td>
<td>this study</td>
</tr>
<tr>
<td>pHH-P(_{\text{hybrid}})-GFP-P2-ipsA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pHH-P(_{\text{hybrid}})-GFP-P3-ipsA</td>
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<tr>
<td>pHH-P(_{\text{hybrid}})-GFP-P4-ipsA</td>
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</tr>
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<td>pHH-P(_{\text{hybrid}})-GFP-P5-ipsA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pHH-P(_{\text{hybrid}})-MIOX-P1-ipsA</td>
<td>pHH01K with MIOX expressed from hybrid promoter; IpsA expressed from constitutive promoters P1-P5</td>
<td>this study</td>
</tr>
<tr>
<td>pHH-P(_{\text{hybrid}})-MIOX-P2-ipsA</td>
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<tr>
<td>pHH-P(_{\text{hybrid}})-MIOX-P3-ipsA</td>
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<td>pHH-P(_{\text{hybrid}})-MIOX-P4-ipsA</td>
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<tr>
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<td>pTrc-Inol</td>
<td>pTrc99A with <em>S. cerevisiae</em> INO1</td>
<td>40</td>
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<tr>
<td>pRSFDCarb-IN-Udh</td>
<td>pRSFDuet with carbenicillin resistance in place of kanamycin resistance, <em>S. cerevisiae</em> Ino1, and <em>A. tumefaciens</em> Udh</td>
<td>this study</td>
</tr>
</tbody>
</table>
4.3 Results

4.3.1 Construction of a MI biosensor

In order to connect MIOX expression to the concentration of myo-inositol (MI), we desired a biosensor to detect MI and regulate translation or transcription of MIOX. The ligand-responsive transcription factor, IpsA, natively regulates MI and cell wall biosynthesis in Corynebacterium glutamicum. It has been shown to bind with high specificity to MI, as well as to multiple promoter regions in the C. glutamicum genome. In C. glutamicum, IpsA significantly activates expression of myo-inositol-phosphate-synthase (INO1), and INO1 is repressed in the presence of MI. IpsA recruits RNA polymerases to the promoter region of INO1, and the presence of MI causes the MI-IpsA complex to dissociate from the DNA, preventing polymerase recruitment. IpsA was also found to upregulate cg0044, a putative ABC transporter, and downregulate inositol catabolism genes, O-succinylbenzoic acid-CoA ligase (menE), and iron homeostasis factors. The first attempt to directly import these IpsA-regulated promoters into E. coli yielded no expression. Next, we engineered a hybrid promoter (Phybrid) that contained -35 and -10 regions recognizable by the native E. coli σ70 RNA polymerase, and an IpsA DNA binding site to permit IpsA-mediated transcription (Figure 17A). We placed the IpsA binding site between the -35 and -10 regions, as it has been shown that this location leads to the tightest repression. In this system, IpsA represses transcription from the hybrid promoter, presumably by blocking access to RNA polymerase. Binding of MI to IpsA causes the complex to unbind from DNA and transcription to proceed. This system may be used to exercise MI-inducible control of expression over any gene placed downstream of the hybrid promoter. Exact promoter and IpsA binding site sequence are given in the Materials and Methods section of chapter 4.

For all IpsA-related work, we utilized plasmid-based expression. For initial characterization of the biosensor, we placed GFP under control of Phybrid and measured the GFP fluorescence output at a range of MI concentrations. For additional tunability, the amount of IpsA in the circuit was modulated by utilizing promoters of different strengths (denoted P1-P5), obtained from the Anderson promoter library, to drive IpsA expression. Sequences and part IDs of the various Anderson promoters used are given in Materials and Methods of chapter 4. We measured the strengths of promoter P1-P5 by driving RFP expression (Figure 18A). When these different promoters were used to drive IpsA expression, we obtained five different circuit variants that achieved different IpsA expression rates. We tested these different circuit variants by measuring endpoint GFP fluorescence when cells were grown with different amounts of exogenously added...
MI. Fluorescence measurements revealed a dose-dependent response to MI (Figure 18B), with increasing MI concentrations leading to increased fluorescence. The presence of multiple knobs for tunability allows flexibility in reaching similar GFP levels through different circuit routes.

**Figure 18.** (a) Endpoint RFP fluorescence when different promoters from the Anderson promoter library were used to drive RFP constitutively. (b) Endpoint fluorescence in cells harboring the IpsA circuit driving GFP expression. P1-P5 refer to different promoters driving IpsA expression in these different circuit variants. Data points are fitted to a dose-response transfer function to yield smooth curves. The “No IpsA” control contains no IpsA and is expected to have constitutive GFP expression from P_{hybrid}, while the “No GFP” control contains only an empty vector.

In addition to evaluating the circuit with exogenously added MI, we tested whether a response can be triggered from intracellularly produced MI. As part of the previously constructed heterologous pathway for glucaric acid production in *E. coli*, myo-inositol phosphate synthase (INO1) from *Saccharomyces cerevisiae* facilitates the conversion of glucose to MI\(^{40}\). We utilized this step of the pathway to intracellularly produce MI from fed glucose and induce GFP expression. Compared to the control strain harboring an empty plasmid, cells expressing INO1 demonstrated a marked increase in fluorescence, depending on the expression level of IpsA (Figure 19A). Consistent with the trends observed with externally-added MI in previous trials, increased IpsA expression decreased fluorescence, even while all strains produced approximately 1.5 g/L MI (Figure 19B). Thus, circuit variants with increased IpsA concentration require more MI to achieve the same GFP output as circuits with weaker IpsA expression. This collection of genetic circuits (P1-P5), with a tunable range of production responses, provided a variety of switching rates to induce MIOX in the glucaric acid pathway.
Figure 19. Circuit behavior with intracellularly produced MI. (a) Endpoint GFP fluorescence in strains containing the MI-responsive circuit driving GFP expression. P1-P5 refer to circuit variants in which constitutive promoters of different strengths drive IpsA expression. For each variant, we collected data for fluorescence data for strains that did or did not contain INOl1. (b) Endpoint MI measured in the culture for strains containing different circuit variants. Error bars represent s.d. of triplicate trials.

4.3.2 Glucaric acid production with delayed MIOX induction

Previous work in the Prather lab has demonstrated that MIOX has a higher activity in the presence of MI\(^40\), presumably because MI helps to stabilize and solubilize MIOX in the cytoplasm. As such, experiments in which MIOX expression was induced at intermediate points in the fermentation (instead of at inoculum) showed a boost in endpoint glucaric acid titers above those obtained from cultures in which MIOX was induced at inoculation\(^33\). We sought to imitate this delay in MIOX induction in an autonomous fashion using the MI biosensor to drive MIOX from P\(_{\text{hybrid}}\). The switching time of this circuit to induce MIOX expression is a joint function of MI and IpsA concentration, and expression of MIOX is delayed until sufficient MI is produced by INOl to unbind IpsA from DNA. Increasing IpsA concentration would increase the concentration of MI required to unbind IpsA.

To prepare strains for glucaric acid production, the genes \(\text{uxaC}\) and \(\text{gudD}\), which encode for enzymes that metabolize glucaric acid and its intermediates, were deleted from MG1655(DE3) strains. These strains were co-transformed with biosensor plasmids in which MIOX was driven by P\(_{\text{hybrid}}\) and IpsA was expressed at various constitutive levels from P1-P5, as well as pRSFDCarb-IN-Udh. To construct a control, we co-transformed plasmid pHH-P\(_{\text{hybrid}}\)-MIOX (no IpsA present; constitutive expression of MIOX from P\(_{\text{hybrid}}\)) instead of the biosensor plasmids, with pRSFDCarb-IN-Udh. Glucaric acid production trials were carried out in T12 medium in shake flasks. Delayed MIOX expression mediated through the IpsA biosensor improved glucaric acid titers up to 3-fold compared to the constitutively-expressed MIOX control (Figure 20). Strain P4 produced the highest titer, presumably because it is closest to the optimal switching time for MIOX in this pathway. Inducing MIOX too early in the fermentation may lead to MIOX instability due to
lack of MI and lower overall conversion, whereas delaying MIOX expression for too long would allow insufficient for conversion of MI. Thus, an intermediate switching time may optimize titers of glucaric acid.

Figure 20. Glucaric acid production in strains where MIOX expression was delayed using the IpsA-mediated MI biosensor circuit. P1-P5 represent circuit variants with different promoters driving constitutive IpsA expression.

4.3.3 Layering multiple dynamic regulation strategies for glucaric acid production

While the MI biosensor allowed delayed induction of MIOX to improve glucaric acid titers, it was at a completely different metabolic control point than Pfk-1. The genetic circuits used to achieve dynamic control at both points were also orthogonal. Induction of MIOX occurred through accumulation of MI and subsequent induction of Phybdid, while downregulation of Pfk-1 occurred through accumulation of AHL and deactivation of P_{osat}. Therefore, these two cases provided two independent strategies to harness dynamic flux control to improve pathway productivity (Figure 21). We hypothesized that instituting both strategies in the same strain may allow synergistic improvements in titers beyond any strategy alone. Few to no studies have explored synergistic layering of multiple heterologous dynamic regulation strategies in the same production strain.

We implemented both strategies in the same strain to measure the effect on glucaric acid titers. As both dynamic modules were tunable with respect to switching times, each could be triggered at different times over the course of the fermentation. We sought to determine which switching times may be optimal for each individual module in this “double dynamic regulation” scenario. Therefore, MI biosensor plasmids (controlling MIOX) and pRSFDCarb-IN-Udh were transformed into strains IB1379GA, L19GA, L24GA and L31GA. In each of these four strains, Pfk-1 was downregulated at different rates due to differential AHL production rates. IB1379GA was the static control strain in which Pfk-1 was not downregulated. In
addition, five different variants (P1-P5) existed for the MI biosensor plasmids in which IpsA expression level differed between variants. In addition, a “no IpsA” control plasmid which contained constitutive MIOX expression from Pbybrid was also included. With 6 different biosensor plasmid variants (P1-P5 + no IpsA) and 4 different QS module variants, we obtained a total of 24 different switching-time combinations between the two dynamic regulation modules. Glucaric acid production trials were conducted on all 24 combinations to determine the best combination of switching times to maximize glucaric acid titers. Endpoint titer data showed that intermediate switching times from both modules were ideal (Figure 22). Specifically, the P2 variant of the biosensor circuit series transformed into L19GA of the QS series produced ~1.8 g/L of glucaric acid. This is the highest glucaric acid titer reported to date in *E. coli* K strains. It is important to note that while P4 led to the highest glucaric acid titer when the biosensor functioned alone in a strain, in the “double dynamic regulation” architecture, P2 was the variant that combined with QS strains best. This indicates that there may be interactions and synergies between the two dynamic regulation strategies to improve titers.

![Figure 21. Schematic depicting the two points in metabolism at which dynamic gene regulation mechanisms are installed to improve glucaric acid production. Dynamic downregulation of Pfk-1 is achieved through the QS-based module discussed in chapter 2 and 3 of this thesis. Dynamic induction of MIOX is achieved through the MI biosensor described earlier in this chapter. Both mechanisms were instituted in the same strains to achieve synergistic improvements in titers.](image)

In order to evaluate the scalability of this approach, production trials for a subset of strains were taken to benchtop bioreactors. Specifically, L19GA containing the P2 biosensor plasmid (pHH-P
hybrid-MIOX-P2-
ipsA) was evaluated since it provided the highest titers among the “double dynamic regulation” strain
combinations. In addition, IB1379GA containing the “No IpsA” biosensor plasmid (pHH-P_{hybrid-MIOX}) was tested as a “static” control and did not contain either of the dynamic regulation mechanisms. Endpoint glucaric acid titers showed that performance in bioreactors was consistent with that observed in shake flasks (Figure 23). The best strain for “double dynamic regulation” produced nearly 2 g/L of glucaric acid while the static strain produced ~0.4 g/L.

Figure 22. Glucaric acid production in the “double dynamic regulation” scenario where Pfk-1 is dynamically knocked down through the QS-based module, and MIOX is dynamically induced through the MI biosensor. Optimal switching times for each module in this architecture were elucidated by testing all relevant combinations. “No IpsA” refers to the biosensor plasmid that does not contain IpsA and therefore has constitutive expression of MIOX from P_{hybrid}. IB1379GA contains no Pfk-1 knockdown as Pfk-1 is under its native promoter. The arrow point to the white bar denotes the fully static control strain which did not contain either of the dynamic regulation mechanisms. Error bars denote s.d. of triplicate cultures.
4.4 Discussion

This work demonstrates that dynamic regulation strategies can be combined in synergistic manners to exercise multiple layers of control of gene expression in the same strain. This is advantageous when multiple points of control exist in metabolism where dynamic modulation of pathway fluxes can boost titers of the target compounds. In the case of glucaric acid production in *E. coli*, two obvious points of control existed to implement dynamic gene regulation. The first was at the G6P branchpoint where competition between glycolytic and heterologous enzymes could be more aptly balanced by dynamically switching off expression of Pfk-1 and redirecting carbon into production formation at intermediate points in the fermentation. This was accomplished by instituting an autonomous QS-based system discussed earlier in this thesis. The second point of control was at the second heterologous step of the glucaric acid production pathway at MIOX. Delaying induction of MIOX expression until its substrate, MI, had accumulated was hypothesized to boost MIOX activity and lead to improved endpoint titers. Upon instituting each mechanism independently, each had shown an improvement in glucaric acid titers over corresponding controls. Combining both strategies in the same strain led to significant titer boosts beyond those obtained by each strategy alone. As the optimal switching time for each strategy was unknown, a combinatorial approach was taken to test all combinations of switching times for each of the two modules. An optimal strain was obtained that produced nearly 2 g/L of glucaric acid, a 5-fold boost over the static strain that did not contain any of the two dynamic regulation devices. Performance of this strain was consistent in benchtop bioreactors as well, indicating that these dynamic regulation modules may be robust in scale-up and scale-down models.
It is important to note that the putative reasons for why dynamic regulation at each of the two control points may improve titers were very different. In the case of the G6P branchpoint, direction of carbon flux was supposed to trade off biomass production for production formation. In the case of MIOX, buildup of its cognate substrate is believed to boost enzyme activity. In both cases, however, dynamic gene regulation was necessary albeit for very different mechanistic reasons.

As we institute more layers of dynamic control, we strive to progress towards the concept of “just-in-time” transcription\textsuperscript{15} that already exists in nature. Zaslaver et al showed that for amino acid biosynthesis pathways, native regulation mechanisms are critical to ensure that enzymes are expressed at optimal times, such as when their cognate substrate pools have built-up\textsuperscript{15}. In addition, the optimal expression level of the enzyme is also controlled precisely, such that those at the beginning of the pathway have higher expression levels. This level of complex regulation minimized metabolic burden and maximized production yield of amino acids. We envision reaching similar levels of complexity and precision for dynamic control of heterologous and engineered bioproduction pathways. Our work in this thesis revolved around developing and characterizing initial strategies to exercise tunable and autonomous dynamic control of pathway enzymes. Developing additional mechanisms, both pathway independent and pathway-dependent, would build the repertoire of strategies available for strain engineers to accomplish target bioproduction objectives. In addition, modulating tuning capabilities of each module in multiple dimensions (beyond trigger time) may offer additional mechanisms of control. As shown in our work, combining dynamic modulation strategies can provide significant boosts in strain performance and it would be very interesting to combine these strategies with traditional strain engineering to determine if we can surpass current performance levels.
Chapter 5

Conclusions and future directions
Abstract

The aims of this thesis were to develop a pathway-independent strategy to autonomously and dynamically control target gene expression, evaluate this strategy in different contexts to determine its industrial applicability and portability, as well as layer it with other strategies for dynamic pathway regulation. This chapter summarizes the findings in this thesis work and proposes directions for future work.
5.1 Thesis summary

In this thesis, systems for autonomous control of pathway fluxes were developed and were shown to improve titers of a variety of target compounds. Specifically, a QS-based genetic circuit was constructed to modulate expression of any target gene. It was used to dynamically downregulate \textit{Pfk-1} and glycolytic fluxes to yield notable improvements in MI and glucaric acid titers. It was then ported to a completely different pathway to improve accumulation of shikimate by downregulating shikimate kinases, indicating its portability and generalizability. With encouraging results in characterization and evaluation studies, this device can potentially be used to dynamically modulate gene expression in any number of cases where dynamic pathway regulation may be advantageous. Potential industrial applicability of our genetic device was kept in mind as we designed evaluation studies and experiments. The key findings in this study are summarized below.

5.1.1 Construction of a pathway-independent strategy to autonomously modulate gene expression

The construction and basic evaluation of various dynamic regulation strategies is presented in chapter 2. Phosphate starvation-based genetic circuits were first characterized to downregulate \textit{Pfk-1} expression in response to the consumption of phosphate in the medium. This strategy, however, was found to have unknown effects on metabolism that confounded final results, presumably since a critical nutrient like phosphate was limiting. The Esa QS system and its \textit{P}_{sall} promoter was then used to create a circuit to allow post-translational knockdown of target genes, such as \textit{Pfk-1}; however, leaky expression of \textit{SspB} led to premature knockdown in \textit{Pfk-1} activity. Finally, a different architecture of the Esa QS system with the \textit{P}_{esa5} promoter was used to construct a circuit to allow transcriptional knockdown of any target gene. In order to attain tunability of switching times, the AHL production rate was modulated by tuning the constitutive expression rate of the AHL synthase, \textit{Esal}. This circuit was first characterized by dynamically turning off expression of GFP at various switching times. It was then applied to glucaric acid production by downregulating \textit{Pfk-1} and glycolytic flux. Production trials showed dynamic downregulation of \textit{Pfk-1} at an intermediate switching rate (due to an intermediate AHL production rate) led to a 20% boost in MI titers over the static strain. \textit{Pfk-1} transcript levels and protein activities were measured at various timepoints to ensure that with higher AHL production rate (through higher \textit{Esal} expression), knockdown in \textit{Pfk-1} expression occurred quicker.

5.1.2 Evaluating robustness and portability of the QS-based device for transcriptional control

With an application-focused mindset, we sought to follow a development workflow to evaluate and characterize the industrial applicability of the QS-based transcriptional control device. Evaluations in
various medium compositions demonstrated that with richer and more industrially relevant medium, the titer boosts obtained from dynamic downregulation of Pfk-1 over the static control strains increased progressively. In addition, curbing glycolytic flux early enough in the fermentation prevented excess production of acetate and an excessive drop in culture pH in shake flasks. In addition, trends seen for MI production with dynamic regulation also extended to glucaric acid production, confirming the initial hypothesis that dynamic downregulation of glycolytic flux would improve glucaric acid production. Scalability analysis to measure device performance across different culture configurations showed that trends were remarkably consistent across the three scales tested (1-ml microplate, 50-ml shake flasks, and 1.25 L bioreactor). With pH control in bioreactors, excess accumulation and pH drop seen with static cultures in shake flasks was mitigated due to the setpoint controls. Instead, higher biomass was observed as acetate was reassimilated into biomass in the static strains. Finally, in order to demonstrate the portability and generalizability of the QS-based module, it was imported into a completely new pathway context where it was used to dynamically downregulate the essential aromatic amino acid biosynthesis pathway to accumulate shikimate. This was the first demonstration of shikimate accumulation in minimal medium without requiring supplementation of aromatic amino acids. With these results, it was encouraging to note that the QS module is applicable across different scales and contexts, indicating that it may be suitable for further evaluation studies in more industrially relevant pathways and production conditions.

5.1.3 Layering multiple dynamic regulation devices to allow complex dynamic pathway control

With a robust, pathway-independent device to modulate gene expression and metabolic fluxes, we sought to implement more complex levels of dynamic control on metabolic pathways. Work in the Prather lab has led to the identification of a MI-biosensor that can turn ON gene expression from a cognate promoter. The transcriptional factor, IpsA, and its binding sequence were imported from C. glutamicum. The binding sequence was used to engineer a hybrid promoter that could function in E. coli and would be repressed by IpsA in the absence of MI. Using exogenously added and endogenously produced MI, the functionality of the constructed biosensor circuit was established with GFP. Furthermore, the biosensor circuit was used to control MIOX so that its expression could be delayed until sufficient pools of MI have accumulated during a production run. Glucaric acid production trials demonstrated that autonomous delay in MIOX expression mediated by this biosensor could improve titers by more than 2-fold. Combining both the QS-based Pfk-1 downregulation and biosensor-based delay in MIOX induction led to the production of ~2 g/L of glucaric acid, the highest titer achieved to date in E. coli K strains. This titer was a 5-fold improvement over the static strains that did not contain any dynamic regulation. Scale-up studies in benchtop bioreactors showed that this synergistic boost in titers was consistent across scales. While this work demonstrated the layering of a pathway-independent and pathway-dependent device, it promoted the concept of layering multiple
dynamic regulation devices to capture synergistic effects for product formation. As more independent devices for dynamic regulation are constructed, layering them together would become more advantageous in cases where multiple points for dynamic control may exist.

5.2 Future directions

5.2.1 Constructing additional pathway-independent devices for dynamic gene regulation

Optimal expression profiles for heterologous pathways must account for a range of factors, including metabolic burden, affinities of enzymes to various intermediates, substrate/product toxicities and enzyme stability and solubility. It is conceivable that bioproduction pathways may have several of these complexities, indicating that multiple points of potential dynamic control may exist to boost pathway productivities. The glucaric acid pathway was one example where dynamic control was advantageous at multiple points. In order to address these needs however, it is necessary to develop more modules for dynamic, autonomous gene regulation.

Quorum sensing provides additional options for systems that may be developed into regulation devices. The LuxS/LsrR system based on autoinducer-2 (AI-2) may be used in conjunction with the Esa QS system as these two systems represent two different families of quorum sensing. The AI-2 system has been utilized in previous studies to allow recombinant protein expression from E. coli, and has been shown to be readily tractable to engineering. This system is distinct from the AHL-based systems in that the signaling molecule itself, AI-2, is structurally different from AHL. AHL contains a lactone ring to which variable acyl branches may be attached. AI-2 on the other hand, contains a boron-mediated ring. Further, it relies on export of synthesized AI-2 and a subsequent import-phosphorylation step in which the incoming AI-2 is phosphorylated and activated. The cognate transcription factor, LsrR, is also different from those in the AHL-based systems. This system, therefore, may be developed as an orthogonal system that could function in parallel with our AHL-based Esa regulation module. One drawback of the AI-2 system, however, is that transport of AI-2 across cellular membranes occurs through transporter proteins, leading to potential heterogeneities within the population. On the other hand, AHL molecules are usually freely diffusible across cell membranes leading to higher likelihood that intra- and extracellular concentrations will be similar. Furthermore, AI-2 is also unavailable in purified form commercially, making basic development testing challenging for researchers. Previous reports have synthesized and purified AI-2 that was subsequently used in characterization experiments.
5.2.2 Developing mathematical models to describe device behavior

As tools for synthetic biology are developed, a drive for standardization is also arising. Researchers are striving to describe device performance with transfer functions so that performance of constructed genetic circuits may be predictable, automated and consistent. These mathematical structures would greatly facilitate the standardization of synthetic biology towards becoming a platform science which may be harnessed for a variety of applications.

Currently, genetic part performance is commonly described qualitatively using empirical data in defined contexts. Porting these behaviors into new contexts, however, often leads to context-dependent variations in performance and ends up requiring new rounds of basic characterizations and testing in the new context. Mathematical models describing genetic circuits are paramount in building better understanding of behavior and its context dependencies. We envision these models to be a mix of mechanistic and data-derived contexts so that they may adequately capture complex circuit behavior in different scenarios. Such models would be especially useful for standardized synthetic biology modules, such as the QS module presented in this thesis which may be applied in different pathways, culture scales and organisms. The process of building a mathematical model itself would improve understanding of how the various parts connect together to render the module performance we observe. This process may first involve characterizing each parts individually, and then systematically connecting them together. For QS, existing mathematical models may be very useful and could be used as a starting point for further complexity. In this thesis, previous models based on the Lux QS system were used to provide basic mathematical structure and Esa-specific parameters were obtained from empirical studies to construct a rudimentary model. More thorough characterization is needed to make the model quantitatively predictive.

In addition, mathematical models for synthetic biology devices would allow simulation of large numbers of contexts and scenarios in silico, instead of requiring manual experimentation. This would not only save valuable time and resources, but may also allow researchers to explore new concepts, applications, and regimes to apply synthetic devices to, especially since computational costs may often be far lower than experimentation. As more quantitative mathematical functions are being developed for individual genetic parts, the next steps involve combining multiple parts together to construct models for whole modules.

5.2.3 Porting to new organisms

As synthetic biology strives to become more translatable to real-world applications, it is becoming increasingly necessary to explore functionality of genetic circuits in new hosts and organisms. E. coli and S. cerevisiae have served as the traditional workhorse laboratory organisms as they have been highly
adapted to laboratory conditions so that they have high growth rates and strong protein production capabilities\textsuperscript{47}. Extensive genetic engineering toolkits have been designed for these organisms to allow complex genetic manipulations. It is becoming increasingly clear, however, that these species are not always the best chassis in different applications, and new organisms are now being explored for different applications. For example, \textit{Bacteroides} is highly efficient for microbiome applications\textsuperscript{88}, \textit{Klebsiella} is studied for nitrogen fixation\textsuperscript{89}, and Chinese Hamster Ovary (CHO) cells are used in biopharmaceutical production of biologics. Often, porting devices to new organisms leads to undesired variability in performance. RNA polymerases and transcription factors may not preserve their affinities for DNA elements\textsuperscript{7}, and variability in sigma factors may hamper genetic device functionality.

Translation of devices and metabolic engineering concepts developed in \textit{E. coli} or \textit{S. cerevisiae} to these new organisms would either require the development of extensive host-specific genetic engineering toolkits, or devices composed of host-independent genetic parts. The former methodology is being explored for specific organisms with wide applicability, such as \textit{Bacillus subtilis} and \textit{Pseudomonas putida}. The latter methodology has been initially explored, and is regarded as the ‘holy grail’ of synthetic biology since it would significantly increase the applicability and valuation of each genetic device developed. Initial efforts to develop such methodologies have revolved around using host-independent parts to construct circuits in intelligent configurations to reduce performance variability.

With these significant challenges, it is vital to determine the organismal portability of a given synthetic biology device, especially one that is developed for wide applicability. The QS device developed in this thesis is pathway-independent and autonomous, and should be applicable in any scenario where dynamic gene modulation is advantageous. As most applications may be outside of \textit{E. coli}, it is vital to determine its portability in new organisms. The first type of new organisms may be other industrially-relevant bacterial species, such as \textit{Corynebacterium glutamicum}, which may be able to utilize the imported parts of the Esa QS system. Eventual translation to eukaryotic and fungal species may also be necessary and may require new host-specific parts.
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Appendix 1

Kinetic Model of QS-based Dynamic Switching at the G6P Branchpoint

Dynamic Mass Balances on Metabolites

The model for dynamic knockdown of Pfk-1 was adapted from Brockman et al. and implemented in MATLAB. The structure of the model was centered around the glucose-6-phosphate (G6P) branchpoint shared by glycolysis (Pgi and Pfk-1) and the gluarcic acid pathway (INO1) in a strain in which zwf has been deleted. Kinetic parameters for enzymes included in the model are given in Table 7, as are the kinetic equations utilized. Enzyme kinetics for these four reactions were based on standard Michaelis-Menten forms for reversible and irreversible enzyme reactions, as suggested in Chassagnole et al. and summarized below:

Irreversible two substrate Michaelis-Menten:

\[ v = \frac{v_{\text{max}}E S_1 S_2}{K_{IS_1} K_{MS_2} + K_{MS_1} S_2 + K_{MS_2} S_1 + S_1 S_2} \]

Reversible Michaelis-Menten:

\[ v = \frac{v_{\text{max}}E S_1 - \frac{S_2}{K_{eq}}}{S_1 + K_{MS_1} \left( 1 + \frac{S_2}{K_{MS_2}} \right)} \]

where S1 and S2 are the respective substrates and E is the enzyme level (scaled to 1 for wild-type levels of enzyme). K_{IS_1} is the equilibrium dissociation constant of S1, but was set equal to K_{MS_2}, as in the irreversible two-substrate Michaelis-Menten equations used in Chassagnole et al. Values for steady-state G6P and fructose-6-phosphate (F6P) levels, cofactor pools, and glucose uptake rate were taken from Chassagnole et al. and were used to calculate the v_{\text{max}} for each enzyme. Further, the v_{\text{max}} for INO1 was calculated based on activity measurements in crude cell lysates and total protein levels.

These calculated v_{\text{max}} values were then utilized in the mass balances of G6P and F6P, as summarized below, to calculate the dynamic profile of these metabolites, such as when Pfk-1 levels were knocked down.

\[ \frac{dG6P}{dt} = v_{pts} - v_{pgi} - v_{ino1} - F_{Pfk} \nu G6P \]
\[ \frac{dF_{6P}}{dt} = v_{p_{6P}} - F_{pfk} \mu F_{6P} \]

\( F_{pfk} \) denotes the fractional Pfk-1 activity at a given time, with the maximum being 1 when there is no Pfk-1 knockdown. \( \mu \) represents growth rate of the culture.

**Autonomous Knockdown of Pfk-1 from QS-based AHL buildup**

A mass action kinetics-based approach for production and degradation of AHL was used, as implemented in previous quorum sensing models\textsuperscript{86}. Further, a standard carrying capacity model was utilized to describe the biomass profile in the culture. As a first order approximation, the effect of different Pfk-1 activity levels on growth rate and final biomass was assumed to be linear. Moreover, the expression of Pfk-1 (and \( F_{pfk} \)) is knocked down based on the buildup of AHL. The mathematical structure of the effect of AHL on \( F_{pfk} \), and the cumulative dissociation constant (\( K_d \)), are taken from Shong et al\textsuperscript{46} which showed that the cumulative response of EsaR-regulated promoters was non-cooperative with respect to AHL concentration, and can be modeled as a standard repression response with a Hill coefficient of 1. The equations summarizing QS-based culture dynamics are summarized as follows:

Cellular growth:

\[ \frac{dN}{dt} = F_{pfk} \mu N \left(1 - \frac{N}{F_{pfk}N_m}\right) \]

AHL balance:

\[ \frac{dAHL}{dt} = k_{AHL}N - d_{AHL}A \]

Fractional Pfk-1 activity:

\[ \frac{dF_{pfk}}{dt} = \left(\frac{K_d}{(K_d + AHL)^2}\right) \ast (k_{AHL}N - d_{AHL}A) \]

where \( k_{AHL} \) and \( d_{AHL} \) are the production and degradation rates of AHL, respectively, as presented in You et al\textsuperscript{86}. The maximum biomass value, \( N_m \), was set to 10\(^9\) cells/mL to correspond approximately to the expected biomass when fed 10 g/L glucose in minimal media. As biomass increases, it leads to greater AHL production and decline in fractional Pfk-1 activity. This feeds back to decrease the growth rate. As \( F_{pfk} \) decreases over time, the steady state pools of G6P and F6P increase, thereby increasing the flux through INO1 over the course of the run. The flux through INO1 can be integrated over time and scaled by the
biomass profile in the culture to give a prediction of the total MI titers at the end of the fermentation. This model was constructed to provide a basis for qualitative trends expected from experimental implementation of dynamic regulation for MI production. Further measurements and parameter characterization can be performed to make the model precisely predictive. Model results are given in Figure 5.

Table 7. Kinetic rate forms and parameter values utilized in the simulation of QS-based control of Pfk-1 activity.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Kinetic equation</th>
<th>Parameters</th>
<th>Value</th>
<th>Source</th>
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<td>Pgi</td>
<td>Reversible Michaelis-Menten</td>
<td>$K_{MPgi,G6P}$</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>$K_{MPgi,F6P}$</td>
<td>0.078</td>
<td>91</td>
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<tr>
<td></td>
<td></td>
<td>$K_{eq}$</td>
<td>0.3</td>
<td>92</td>
</tr>
<tr>
<td>INO1</td>
<td>Irreversible two substrate Michaelis-Menten</td>
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<tr>
<td></td>
<td></td>
<td>$K_{MIno1,NAD}$</td>
<td>0.008</td>
<td>94</td>
</tr>
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<td>Pfk-1</td>
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<td></td>
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<td>86</td>
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<tr>
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<td>N/A</td>
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<td>$1.9 \times 10^{-4}$</td>
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