Coupling feedback genetic circuits with growth phenotype for dynamic population control and intelligent bioproduction

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Abstract

Metabolic engineering entails target modification of cell metabolism to maximize cell's production potential. Due to the complexity of cell metabolism, feedback genetic circuits have emerged as basic tools to combat metabolic heterogeneity, enhance microbial cooperation as well as boost cell's productivity. This is generally achieved by applying social reward-punishment rules to eliminate cheaters and reward winners. With metabolite-responsive transcriptional factors to rewire gene expression, metabolic engineers are well-positioned to integrate feedback genetic circuits with growth fitness and achieve dynamic population control. Towards this goal, we argue that feedback genetic circuits and microbial interactions will be a golden mine for future metabolic engineering. We will summarize the design principles of engineering burden-driven feedback control to combat metabolic stress, implementing population quality control to eliminate cheater cell, applying product addiction to reward productive cell, as well as layering dual dynamic regulation to decouple cell growth from product formation. Collectively, these strategies will be useful to improve community-level cellular performance. Encoding such decision-making functions and reprogramming cellular logics will enable metabolic engineers to deliver robust cell factories and pave the way for intelligent bioproduction. We envision that various cellular regulation mechanisms and genetic/metabolic circuits could be exploited to achieve self-adaptive or autonomous metabolic function for diverse biotechnological and medical applications. Applying these design rules may offer us a genetic solution beyond bioprocess engineering strategies to further improve the cost-competitiveness of industrial fermentation.

Keywords: genetic circuits, metabolic engineering, dynamic population control, growth fitness, synthetic biology

Introduction

Metabolic engineering is an enabling technology to produce fuels, chemicals and pharmaceuticals alternative to traditional petroleum-based manufacturing processes. Current industrial-scale fermentation is largely relied on native strains that are derived from natural selection and evolution, and the scope of product is limited to proteins and primary metabolites, including biofuels, amino acids, organic acids and antibiotics [1]. This is because naturally evolved strains tend to maintain genetic stability and phenotypic robustness across a broad range of evolutionary timescale and ecological niches, this important feature provides the basis for fermentation scale up and leads to consistent titer, yield and productivity (TYP). Unlike natural selection, metabolic engineering is the targeted modification of cell metabolism and redistribution of carbon, energy and electrons to maximize the production of a specific compound. With sophisticated genetic toolkits, metabolic engineers have been able to fine-tune gene expression, effectively modify cell metabolism and deliver tailored cell factories with improved TYP index [2]. By engineering heterologous pathways or endogenous metabolism, metabolic engineers have now been able to produce a large portfolio of commodity chemicals [3, 4], novel materials [5], advanced biofuels [6-9], nutraceuticals and pharmaceuticals [10, 11] from low-cost feedstocks. This is often achieved through well-established metabolic engineering strategies including overexpression of rate-limiting steps [12], deletion of competing pathways [13], managing ATP [14, 15] and recycling NADPH and other cofactors [15].

To engineer efficient microbial cell factories with superior TYP index, one has to evaluate the cost and benefits of genetic modifications that are associated with strain engineering process. Any

genetic modification, if not associated with competitive fitness advantage, will inevitably burden the cell with additional energy cost to diminish pathway yield [16, 17]. Evolution has made cell to maximize their proliferation and often conflicts with engineers' interest for metabolite overproduction. These competing interests and unaligned objectives will compound strain engineering effort. This is also exacerbated by the fact that introduced gene clusters are often subject to tight cellular regulation. This will lead to the so-called metabolic imbalance, for example, precursor flux improvement by overexpression of heterologous pathways may not be accommodated by downstream pathways; intermediate accumulation or depletion may compromise cell viability and pathway productivity [18]; and overexpressed heterologous protein may penalize the cell with additional energy cost and elicit cellular stress response [19]. On the other hand, engineered strains with highly intervened regulatory and metabolic networks are most often tested at bench-scale. When translated to real industrial production with complex media and partially-mixed bioreactor conditions, these engineered strains often fail to adapt themselves to the changing environment and maintain metabolic homeostasis [20, 21]. Isogenic cell cultures are generally regarded as phenotypically uniform, while the effects of cell-to-cell phenotypic variation are ignored. However, multiple recent researches demonstrated that the phenotypic variation has been greatly underestimated with regard to diminishing TYP during scale-up [20-22].

Metabolically engineered strains will accumulate unwanted mutations and result in subpopulation of cell competing for the limited energy/substrate resource. This phenotypic variation generally reduces cellular productivity and may lead to completely abortive phenotype if the mutant population escapes, propagates and takes over the entire population during fermentation scale-up

[20]. Phenotypic profiling has demonstrated that the source of metabolic heterogeneity and genetic variation could arise from a multitude of factors, including substrate inhibition, the buildup of toxic intermediary metabolites, product toxicity, nutrient depletion, genetic mobile elements translocation, plasmid instability, evolution pressure, ecological interactions and other stress factors [23-25].

One of the possible solutions is to engineer feedback control genetic circuits and conferring overproduction strain with competitive growth advantage. This competitive fitness would provide a social reward-punishment rule to encourage the growth of the overproduction strain and discourage the growth of the cheater cell (cheater cell could be defined as the degenerated population with compromised TYP index). Enhancing microbial cooperation in such a way would allow the overproduction strain to outcompete the low-production or cheater cell. Evolutionarily, this is a process to incentivize social cooperation and enhance cell's collective interest rather than cell's individual interest. To achieve this goal, it is essential to encode feedback genetic-circuits into living cell factories, so that the engineered cell autonomously adjusts pathway expression to combat metabolic heterogeneity and environmental stress. This selective advantage, if designed properly, would favor the growth of the overproduction cells and eliminate the growth of the cheater cell [26]. Past two years' achievements have witnessed the application of dynamic control theory to maximize pathway efficiency [27-29]. Ideally, a sensor-transducer-actuator system can be used to sense metabolic burden, actuate transcriptional event and compensate for the loss in metabolic activity. Implementation of this strategy would require the engineering of transcriptional factor-based biosensors and informing the cell with decision-making function to selectively favor the growth of the overproduction population and eliminate low-production cells. In this review, we intend to

summarize the recent achievements to engineer feedback genetic circuits that confer the cell with selective advantage toward intelligent biomanufacturing with improved TYP index [16, 20, 22].

Burden-driven feedback control to combat metabolic stress

In synthetic biology, to construct metabolic pathway or synthetic device, it is generally required to assemble multiple gene clusters and protein regulators. However, imposing high dosage of "foreign" proteins without considering host carrying capacity will overload the cell causing burdensome effects to the host, as introduced genetic circuits typically compete with the native pathway for limited cellular resources [30]. As a response to unnatural protein expression burden, engineered cells exhibit decreased growth fitness and reduced TYP index [31]. Inspired by the fact that bacteria may have developed native defense mechanisms to resist the sudden change of gene/protein expression, the authors conducted RNA-seq studies and characterized a number of burden-responsive promoters. The authors revealed that the transcriptional activity of the selected promoters (*ibpAB*, *htpG*, *groSL* and *dnaKJ*) were well-correlated with the level of unnatural burden [27]. For example, promoters related to heat-shock response was rapidly activated upon induction with elevated temperature.

Using the best-performing *htpG1* promoter, the authors built a CRISPR-dCas9-based feedback control system that automatically senses burden signal and adjusts the expression of a synthetic reporter gene (**Figure 1A**). Specifically, the burden-responsive *htpG1* promoter was used to drive the expression of a single guide RNA (sgRNA). When the reporter gene starts causing burdensome effect, the CRISPR sgRNA will direct a catalytically inactive dCas9 to target specific region of the

promoter P_{BAD}, which drives the expression of the synthetic reporter gene. This negative feedback control mechanism provides a robust system to autonomously regulate the reporter gene expression and suppress the burden signal. The constitutive expression of dCas9 enables rapid sgRNA-mediated transcriptional repression to the P_{BAD} promoter. This system was introduced into an *Escherichia coli* cell with overloaded foreign gene constructs, where the burden signal can be monitored in real time [32]. The host cells equipped with this system maintained high capacity for overall protein expression by autonomously tuning the burden signal over a range of induction level. Cells equipped with this feedback controller also maintained robust growth and outperformed uncontrolled cells in terms of foreign protein yield in 24-h batch fermentation (**Figure 1B and 1C**). Using sgRNAs with variable affinities to promoter P_{BAD}, the strength of feedback control system could be tuned to yield the optimal synthetic construct maximizing host capacity for burdensome gene expression. The regulation node can be easily retargeted by altering the guiding sequence of the sgRNA, and multiple nodes can also be regulated by co-expressing multiple sgRNAs. Because the regulation is directed by sgRNA, this control system is faster than others that are based on protein expression. Moreover, because P_{htpG1} is a σ^{32} transcriptional factor-regulated promoter, it is possible to transfer this system to other bacteria. In conclusion, this universal burden-responsive feedback control system is modular, tunable and portable, and offers an intriguing way to maintain robustness and performance under changing and heterologous conditions typically seen in scaled-up fermentation [27].

Engineer population quality control (PopQC) to eliminate cheater cell

Past two decades' achievement in pathway engineering has demonstrated the success to employ

classical stoichiometrically-based approach to improve biosynthetic performance of engineered cell factory. These strategies include enzyme engineering, flux redirection, dynamic regulation, protein scaffold and compartmentalization, *et al* [33-35]. However, both protein abundance and biosynthetic performance differ significantly within isogenic populations [22, 36]. Although this heterogeneity can be helpful for organisms to survive under varying circumstances in natural environment, it is counterproductive in bioprocess development. The low-performing subpopulation cells will consume nutrient and reduce the overall yield. By characterizing this metabolic heterogeneity, the authors observed a more than nine-fold variation in free fatty acid (FFA) production across the isogenic cell culture. Moreover, more than 50% of the total FFA was produced by a minor high-performing population, while the majority of population yielded little products [22].

Based on these observations, a concept of population quality control (PopQC) was proposed [22]. In principle, by utilizing an end product-responsive biosensor to regulate the expression of a survival gene (such as a tetracycline efflux pump encoding gene), PopQC enriches the high-performing population, and effectively reduces low-performing population under a given selection pressure (such as tetracycline) during the bioprocess (**Figure 2A**). The authors successfully applied PopQC to improve the production of FFA and tyrosine using two alternative design principles, which demonstrated the benefits and broad application of PopQC.

In order to apply PopQC design principle in FFA biosynthesis (**Figure 2A**), the FFA-responsive transcriptional repressor FadR was used to regulate the expression of tetracycline efflux pump (TetA) [37]. The DNA binding activity of transcriptional factor FadR was regulated by acyl-CoAs, which were derived from FFA by the *E. coli* native acyl-CoA synthetase (FadD). In the absence of

acyl-CoAs, FadR binds to promoter P_{AR} and represses transcription of efflux pump gene (*TetA*). When FFA was overproduced, the resulting fatty acyl-CoA would deactivate FadR, resulting in consistent expression of the tetracycline efflux pump to combat the tetracycline toxicity. Applying this PopQC design would selectively choose the population with high FFA production and eliminate the population with low FFA production. A similar PopQC design principle was also applied to improve tyrosine biosynthesis (**Figure 2C**), the feedback control circuits consist of transcriptional factor TyrR and its cognate promoters P_{T1} and P_{T2}, respectively. When high level tyrosine is produced, the TyrR-tyrosine complex activates *tetA* transcription, allowing high tyrosine-producing strain to survive the selection pressure (tetracycline). As a result, populations that are more productive in FFA or tyrosine, will be enriched and gradually dominate the population during the fermentation (**Figure 2B**). Both FFA and tyrosine titer and yield improved about three-fold in the strain equipped with PopQC system under selective pressure compared to the strain without selective pressure or PopQC control.

In an alternative design, the antibiotic resistant gene was replaced with essential genes for cell growth (**Figure 2D**). This design removed the use of expensive and environmentally unfriendly antibiotics. For example, the leucine biosynthetic operon *leuABCD* was controlled by FFA responsive promoter P_{AR} [22]. The FFA pathway and PopQC construct were transformed into a leucine-auxotrophic (deleted chromosome *leuABCD*) *E. coli* strain. By coupling FFA production with leucine biosynthesis, this design will create a selective advantage to encourage the growth of high FFA-producing strains and discourage the growth of the low FFA-producing strains. Populations with high FFA titer will outcompete the populations with low FFA titer by synthesizing

more leucine, resulting in better growth fitness in FFA overproduction strains. In bench-top bioreactor, highest FFA titer and productivity were obtained by the strain with PopQC control. Taken together, PopQC system is a powerful and effective approach to eliminating low-performing, nongenetic variation subpopulations, which may also benefit large-scale bioproduction process under industrial settings.

Engineer product addiction to enhance microbial cooperation

Heavily-intervened regulatory network of native strains would inevitably lead to reduced cell fitness and degenerated production, as exemplified by metabolite depletion, toxicities of intermediates and byproducts accumulation in metabolically engineered strains [16, 38]. Due to the conflicting goals between cell growth and metabolite production, there is always an intriguing tradeoff: cells with better growth fitness will dominate the population under evolutionary pressure, resulting in declined production profile and even abortive phenotype. As a result, coupling growth fitness with metabolite production represents an attractive solution for fermentation scale-up and long-term bioproduction. As a comparison, PopQC is an effective method to enrich high-performing subpopulation, however both the use of antibiotics (tetracycline) and efforts to constrain medium composition (leucine-depleted minimal medium) may not be economically viable for large-scale fermentation. By coupling end-product concentration with expression of nonconditionally essential genes, one may engineer cells that are synthetically addicted to the end-product, so the engineered cell can maintain consistent performance to produce this end-product without losing the growth fitness. This has proven to be an effective solution to enrich high-yield subpopulation without the use

of chemically-defined media or supplementing expensive antibiotics.

Recent studies have established three criteria to engineer metabolite-addiction phenotype [20, 21]: (i) the product addiction should be nonconditional, (ii) the product addiction should not reduce product yield or titer, (iii) the product addiction should not reduce cell fitness. To make product addiction nonconditional with cell growth (criterion i), the target gene(s) should be essential for cell growth but not involved in primary metabolism. In a recent study to improve mevalonic acid (MVA) production, operon *folP-glmM* was chosen from a panel of operon candidates [21]. This operon is necessary for cell growth in complex and minimal media. The authors firstly validated the L-arabinose addiction by replacing the native *folP-glmM* promoter with two P_{BAD} variants. P_{BAD} is responsive to L-arabinose via *E. coli* homodimeric AraC protein. AraC represses P_{BAD} transcription in the absence of L-arabinose, while upon L-arabinose binding, AraC-arabinose dimer activates P_{BAD} promoter. By tuning ribosome binding site (RBS) strength, a design with least fitness-cost (criterion iii) but obvious growth-coupling effect was used in the subsequent development of MVA-addicted production strain. A constitutive MVA pathway was introduced to synthesize MVA, and an MVA-responsive variant $AraC_{mev}$ was used to activate P_{BAD} in response to MVA [5, 39]. Finally, the best-performing construct P_{BAD}-RBS-*folP-glmM* was integrated into the chromosome. When a cell produces high level MVA, the $AraC_{mev}$-MVA dimer activates *folP-glmM* expression, which results in high cell growth rate (**Figure 3A**). On the contrary, low MVA production will trap the *folP-glmM* expression at inactivated states, which results in low growth rate that will be falling behind the growth of the high-production strain. The authors validated the growth response in both M9 minimal medium and 2xYT complex medium, demonstrating the addiction was not dependent on nutrient

omissions.

The resulting strain was then used to improve MVA production and understand how evolutionary pressure changes the metabolic profile during fermentation scale-up. Specifically, the fermentation scale-up was simulated by serial passaging lineages of each strain from the master cell bank to large production bioreactors. MVA production and cell growth fitness of the engineered strain were compared to the control strain, which is identical to MVA addiction strain but with wild-type *folP-glmM* promoter. At the beginning of the simulation, both strains displayed equal growth rate (criterion iii) and MVA yield (criterion ii). With serial cell passage, the MVA-addicted lineage retained their initial growth rate throughout the simulation, and remained above 95% of maximal productivity at the end of simulation (**Figure 3B**). However, the control lineage gradually increased maximum growth rate accompanying with decreased MVA productivity, indicating a propensity of population division and drastic shifting of metabolic homeostasis, which could be correlated with the overexpression of multiple genes [20]. The control lineage gradually lost MVA production, which fell to below 5% of maximal MVA titer at the end of simulation (**Figure 3C**). These results demonstrated that product addiction system could function as intended to selectively reward the growth of the high MVA-producing population. The authors further deep-sequenced the pathway populations and discovered enrichment of mobile elements in the non-addicted lineage population, indicating that the product addiction system created a selective advantage to favor the growth of the high MVA-producing cell [20]. This product addiction feedback control system provides an excellent example to genetically prevent cells from losing production, paving the way for engineering more robust and predictable biomanufacturing platforms in the future.

In the PopQc strategy, cells were sorted into high-performing and low-performing populations, and the majority FFA production was contributed by a minor population [22]. In addition, the cell-to-cell variation was believed to be a nongenetic effect, and the phenotype was passed to daughter cell via epigenetic inheritance. However, in simulations of large-scale MVA fermentation, the authors detected multiple genetic mutations in the MVA pathway by using ultra-deep sequencing [20, 21]. This suggests that the phenotypic variation could be a result of both genetic mutation and nongenetic cell-to-cell variations.

Population-based layered dynamic regulation to decouple cell growth with metabolite production

Conferring cell with growth fitness is an efficient approach to improve metabolite production. This strategy may only apply to partially- or non-growth associated product formation. For metabolites that are naturally derived from primary metabolism, it remains a challenging task to balance the tradeoff between cell growth and metabolite production. Addressing this challenge require the dynamic redistribution of carbon flux between cell growth and product synthesis [29]. A recent example of glucaric acid production could best illustrate this point [28]. Specifically, the glycolytic intermediate glucose-6-phosphate (G6P), derived from the native phosphoenolpyruvate-dependent phosphotransferase system (TPS), could enter either the heterologous glucaric acid synthesis pathway or glycolytic pathway to provide precursors for cell growth. In the D-glucaric acid heterologous pathway, G6P is sequentially converted to *myo*-inositol (MI), glucuronic acid, and glucaric acid by activities of *myo*-inositol-1-phosphate synthase (MIPS), endogenous phosphatases, *myo*-inositol oxygenase (MIOX), and uronate dehydrogenase (Udh) [40].

Cell growth and glucaric acid production compete for the same G6P precursor; this fact presents a major challenge to efficiently produce glucaric acid in *E. coli*. To address this challenge, the authors investigated two independent yet complementary strategies to improve glucaric acid production. The glycolysis entry point enzyme, Pfk-1 (phosphofructokinase), competes MIPS (myo-inositol-1-phosphate synthase) for G6P in glucaric acid pathway. Knocking out *pfk-1* would cause a lethal phenotype or significantly reduce the cell growth rate [41]. To effectively redirect carbon flux from glycolysis to glucaric acid production, a pathway-independent quorum sensing (QS) switch was engineered to autonomously tune down the expression of Pfk-1 [29]. QS genetic circuit regulates and controls gene expression in a cell density-dependent manner, primarily through the autoinducer molecule 3-oxohexanoylhomoserine lactone (AHL). In the absence of AHL, transcription regulator EsaRI70V (a mutant version of the original EsaR) binds the cognate promoter P_{esaS} and activates transcription [42]. Accumulation of AHL instead disrupts EsaRI70V binding with DNA and decreases gene transcription from P_{esaS} promoter (**Figure 4**). One of the challenges is to determine the right switching time when P_{esaS} promoter should be activated, so that the carbon flux could be effectively switched from glycolysis to glucaric acid production. Since AHL is produced by AHL synthase EsaI, the switching time of P_{esaS} promoter could be controlled and tuned by modulating EsaI expression, which could be achieved through using a library of promoter and ribosome binding site variants [29].

A second challenge is arising from the unstable nature of MIOX, whose activity declines rapidly in the recombinant cell during the fermentation process. Ideally, it is desirable to turn on the expression of MIOX only when there is enough metabolic intermediate *myo*-inositol (MI) (Shiue,

Eric Chun-Jen, PhD thesis, Massachusetts Institute of Technology, 2014). This constraint necessitates a just-in-time gene expression pattern to avoid wasting of cellular resource and efficiently convert metabolic intermediate to end product. To solve this challenge, the authors have proposed a few engineering strategies to delay the expression of MIOX. Specifically, the MI-responsive transcriptional factor, IpsA, was used to sense the exact amount of MI inside the cell [28]. A hybrid promoter P_{hybrid}, consisting of *E. coli* σ^{70} RNA polymerase recognition regions (-35 and -10 regions) and IpsA DNA binding site, was constructed to translate intracellular MI signal into a transcriptional output. Accumulation of intermediate MI removes IpsA-mediated transcriptional repression and activates the expression of MIOX, which converts MI to glucuronic acid. As a result, *Miox* expression was delayed until sufficient MI was accumulated. To demonstrate the utility of this system and maximize the production of glucaric acid, a collection of MI-responsive promoters with differential transcriptional activity was constructed to control the timing of *Miox* expression. Glucaric acid titer was improved up to 2.5-fold by the strain harboring the optimal sensor-regulator system [28].

Both the QS and MI-responsive promoter systems improved glucaric acid titers when introduced to different chassis strains [29]. To synergistically boost titers, the authors layered these two orthogonal genetic switches and successfully developed an autonomous yet tunable dynamic regulation system (**Figure 4**). The pathway-independent QS-based circuit autonomously down-regulated Pfk-1 and redirected G6P from glycolysis to glucaric acid synthesis, where the shifting of metabolic activity was completely controlled by cell density. The pathway-dependent IpsA-based circuit delayed MIOX expression until the cell accumulated threshold level of MI, where

the switching of gene expression was solely determined by the level of the intermediary MI concentration. Layering these two genetic switches led to highest glucaric acid production improvement, demonstrating the synergistic effect of this layered dynamic regulation strategy [28]. Completion of such a delicate control requires the integration and coordination of a large number of biological parts across multi-dimensional design space, which would not be possible without our improved understanding of cellular control schemes in general and DNA-protein interactions in specific.

Conclusions and perspectives

Recent practice in metabolic engineering demonstrated that metabolic heterogeneity plays a major role in determining cellular performance and metabolic productivity [21, 22, 27, 43]. A robust and reliable biomanufacturing system would require metabolic engineers and synthetic biologists to develop novel strategies to mitigate this metabolic heterogeneity. Traditional bioprocess engineering has been proven effective to maintain both prolific and productive phenotype, which was achieved through modulating environmental or nutritional conditions, including agitation, temperature, pH, dissolved oxygen (DO), dilution rate and feeding of limiting media or antibiotics. These classical approaches are generally found helpful to improve the growth fitness and the metabolic performance of the tested strain in well-controlled lab conditions. However, heavily engineered lab strains will often encounter metabolic burden, enzyme/cofactor imbalance, nongenetic cell-to-cell variation and plasmid instability issues leading to loss of metabolic performance [16]. To address this challenge, it is necessary to seek genetic approaches to enhance the selection processes that maintain metabolic

homeostasis. In principle, this could be achieved through engineering delicate cellular logics that link metabolite production with cell growth fitness.

Fermentation could be viewed as an evolutionary process with productive cells constantly dividing into multiple populations that are deviating or drifting from the optimal setting of the parental population. Engineering more stringent transcriptional or translational control that are resistant to random mutations would help homogenize the production phenotype. Engineering cellular defense systems to increase phenotype plasticity or nucleus multiplicity would be also attractive strategies to combat metabolic heterogeneity. Alternately, we can engineer feedback genetic circuits that allow cells to sense, respond to, and relieve the metabolic burden [27, 44]. We can also apply social "punishment-and-reward" principles to eliminate the non-productive cell and incentivize the productive cell [22]. Most of current studies are based on rewiring cellular logics and the development of transcriptional factor-based biosensors (TFBBs), which provides the framework for directly linking metabolite production with cell growth. For instance, the transcriptional activity of metabolite-responsive promoters (MRPs) could be rewired to drive gene expression that is advantageous to productive cell or deleterious to cheater cell. A non-conditional essential gene and an end-product inducible efflux pump gene have been successfully applied to enhance the cooperation of the productive cell or reduce the fitness of the cheater cell [21].

Despite the great success of feedback genetic circuits to resolve bioprocess problems, we should fully anticipate the challenges in this molecular design and engineering process. First, we need to find synthetic promoter and cognate transcriptional factor pairs for our specific applications. All the systems reviewed above were based on synthetic promoters and inducible transcriptional factors.

However, limited by the number of metabolite-responsive transcriptional factors, not any bioprocess has an opportunity to be equipped with such system [45]. A great hurdle is to profile the large volume of bioinformatic database and characterize the interactions of promoter and transcriptional factor. Thanks to the development of next generation sequencing, it becomes possible to discover vast majority of inducible promoters under specific circumstance. Rational design and random mutation can also be used to change the substrate specificity of the transcriptional factors. A second challenge is the leakage of the inducer or end-product between different cells. This may result in the failure or unintended output of the designed system. Profiling the inducer or end-product distribution among cells is required before integration of the sensor-transducer-actuator system. Cell transporters, including permeases or efflux pumps may be deleted or altered, if metabolite transportation across the entire population causes a problem. A fundamental challenge is how to prevent the mutation of the inducible system. As a practical approach to maintain metabolic performance, one could engineer an “immune” system to make the highly productive cell insensitive to environmental variations. This could be achieved by engineering more efficient DNA-proofreading, correcting activity of the native or non-natural DNA polymerase or the use of epigenetic regulation.

Most of the reported strategies are targeting on nongenetic cell-to-cell variations to confer competitive fitness. Genetic underpinnings that are associated with metabolic heterogeneity remain an untapped area. In addition, creating selective advantages by rewiring cellular feedback control circuits would itself increase burden of gene expression to the engineered cell. One has to evaluate the cost and benefits of imposing such a feedback design [43, 46, 47]. It is necessary to computationally model the population-scale pathway yield and understand the fundamental design

rules how to implement this strategy. It is true that feedback control generally increases system stability and robustness. But it would be problematic if one ignores the cost and benefits of engineering such a genetic circuitry, as the added cellular "logics" will compete cellular resources that would otherwise be utilized by the engineered pathway. Unravelling the fundamental principles of resource competition and decoding biological complexity to build minimal cells would help us move forward how to engineer feedback control genetic circuits into living cell factories. The advance on intelligent cell design will also enable us to engineer smart living therapeutics and probiotics to treat emerging diseases. We envision this will be a fast-evolving area, and an integrated computational and experimental approach incorporating both evolutional concepts and ecological perspectives should be encouraged for further development in this area.

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Author contributions

YL and PX conceived the topic. YL and PX wrote the manuscript with input from SQ.

Conflicts of interests

The authors declared no conflicts of interests.

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Table 1 Examples and metabolic engineering applications of coupling feedback genetic circuits with growth phenotype

Method	Descriptions	References
An *in vivo* monitor to track cell burden	An *in vivo* monitor tracking changes in the cell capacity was developed to assay the burden imposed by synthetic constructs. The monitor was used to identify construct designs with reduced burden.	[32]
Burden-driven feedback control	A burden-driven feedback controller was constructed and used to control the expression of heterologous proteins. This method improved the host cell fitness and total protein production.	[27]
Population quality control (PopQC)	The authors demonstrated that the metabolic heterogeneity was mainly caused by nongenetic cell-to-cell variation. The PopQC method was designed to eliminate low-performing cells and enriching high-performing cells to improve the ensemble production.	[22]
Synthetic auxotrophic system	By using the synthetic auxotrophic system, gene expression was stably maintained for 40 generations with minimized cell-to-cell variation under antibiotic-free conditions. This method significantly improved the production of itaconic acid and lycopene.	[48]
Orthogonal and	The sensor-selector coupled the concentration of muconic acid to	[49]

pH-tunable sensor-selector	cell fitness by using BenM driving an antibiotic resistance gene. The sensor-selector limited the rise of nonproducing cheaters, and was successfully used to selectively enrich for best-producing variants of muconic acid production strains.	
Carbon source utilization based continuous evolution	This method established and sustained growth-production coupling in a medium with maltose as the sole carbon source. This method was more robust with a much lower escape risk than the antibiotic resistance-based circuits.	[50]
End-product addiction system	This method addicted the cell to its end-product to extend the productive life time of engineered strain. This system was validated in both nutrient depleted minimal medium and complex medium.	[21]
Population-based layered dynamic regulation	This method layered two orthogonal, autonomous, and tunable dynamic systems in engineered cell to decouple cell growth with metabolite production.	[28]

Figure and legends

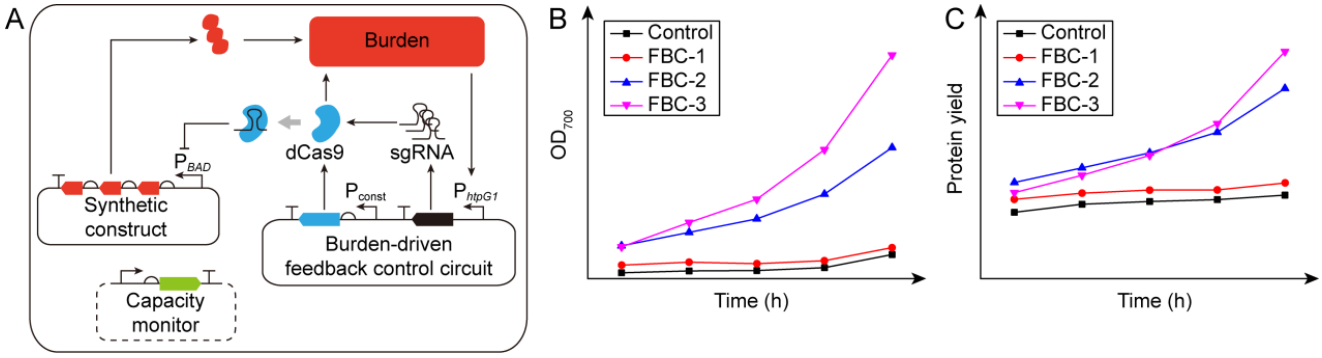

Figure 1. Burden-driven feedback control.

A. Mechanism demonstration of the burden-driven feedback control system. Maintenance and expression of synthetic DNA causes protein overloading and unnatural burden to the host cell. Transcription of sgRNA is controlled by the burden inducible promoter P_{htpG1}. A constitutive promoter is used to drive the expression of the catalytically-inactive dCas9, which is directed by sgRNA to bind to specific region of P_{BAD}. This feedback control system monitors the capacity of the cell and represses the expression of synthetic DNA construct. Burden imposed by synthetic DNA construct was monitored by tracking changes in the capacity of the host cells. **B.** Time course of biomass accumulation by cells equipped with feedback control systems of variable strengths. **C.** Time course of protein yield of synthetic DNA construct by cells equipped with feedback control systems of variable strengths. P_{BAD}, *BAD* promoter; P_{const}, constitutive promoter; P_{htpG1}, *htpG1* burden-inducible promoter; Control, cell equipped without feedback control system. FBC, feedback controller. FBC-1, FBC-2, and FBC-3 refer to the FBCs of variable strengths, which were achieved by using sgRNAs with different binding affinities to P_{BAD} promoter.

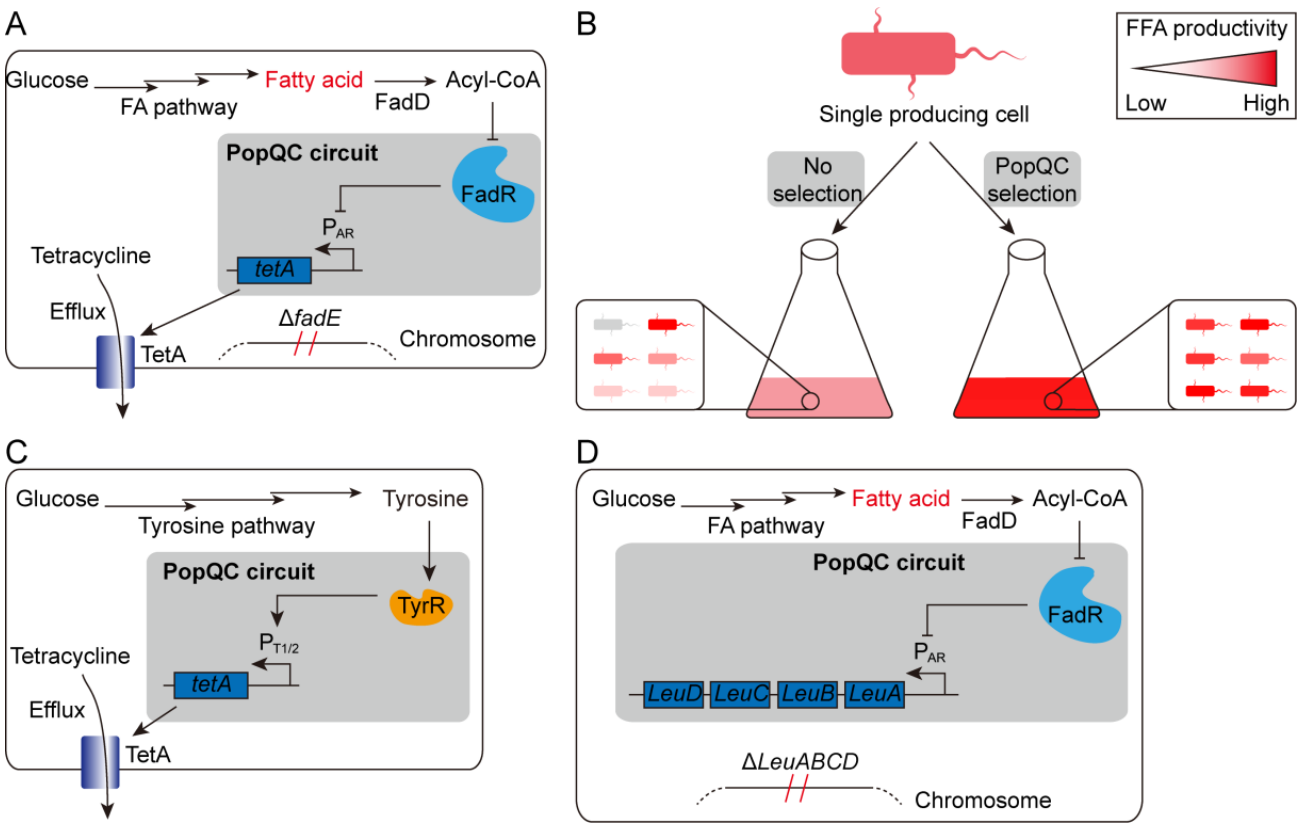

Figure 2. Schematic demonstration of population quality control (PopQC) and its applications.

A. Mechanism of application of PopQC in free fatty acids production. Transcriptional factor FadR responds to fatty acid via fatty acyl-CoA. Transcription of tetracycline efflux protein gene *tetA* is controlled by a synthetic promoter P_{AR}, which is repressed by FadR in the absence of fatty acyl-CoA. The accumulation of fatty acid, which can be converted to fatty acyl-CoA by native acyl-CoA synthetase (FadD), results in FadR deactivation and dissociation from promoter P_{AR}, and thus enable the expression of the *tetA* gene (tetracycline efflux pump) allowing the cell to grow. **B.** In the presence of PopQC and selection pressure, high-performance cells outcompetes the low-performance cell to degrade tetracycline. Predominance of high-performance cells in the population prevents low-performance cells from wasting nutrients and enhances the overall productivity. **C.** Application of PopQC in tyrosine production. Promoters P_{T1} and P_{T2} were constructed to control the transcription of *tetA*. Tyrosine responsive transcriptional factor TyrR, which is responsive to tyrosine, activates P_{T1}

and P_{T2} in the presence of TyrR. The accumulation of tyrosine confers the cell improved growth fitness in the presence of tetracycline. **D.** An alternative PopQC design principle for fatty acid production by using leucine as a selection criteria. In this design, *tetA* was replaced with an essential leucine pathway gene operon *LeuABCD*. The chromosome *LeuABCD* was removed, so that higher FFA productivity will confer the cell higher growth rate by providing more leucine. FadD, fatty acyl-CoA synthetase; FadR, FFA-responsive transcription factor; P_{AR}, promoter repressed by FadR; TetA, tetracycline efflux protein; *tetA*, TetA conding gene; *LeuABCD*, leucine biosynthetic gene cluster.

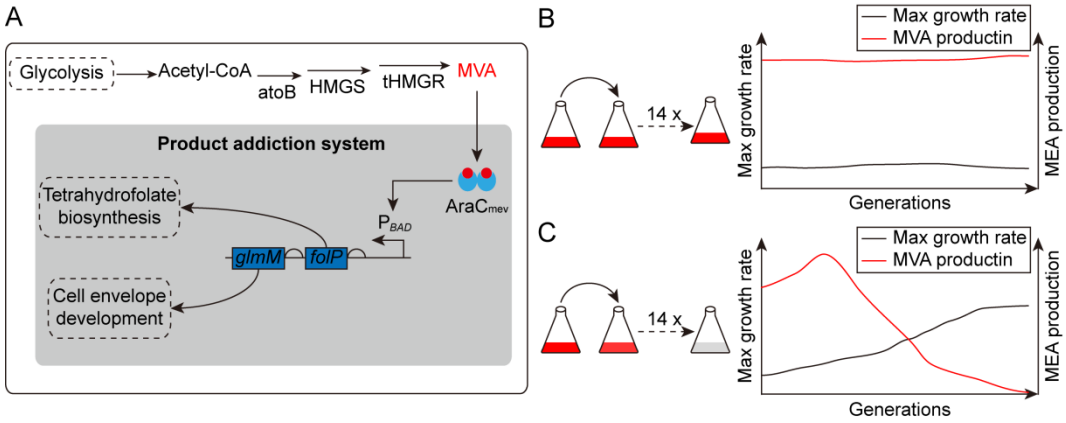

Figure 3. Engineering synthetic product addiction to reward the high-performance cell.

A. Mechanism of synthetic end-product addiction system. $AraC_{mev}$ is a variant of transcriptional factor AraC. $AraC_{mev}$ represses P_{BAD} transcription in the absence of MVA, while it activates P_{BAD} in the presence of MVA. Dihydropteroate synthase (FolP) and phosphoglucosamine mutase (GlmM), involved in tetrahydrofolate biosynthesis and cell envelope development respectively, are both essential for cell growth. This system selectively prefers the growth of cells producing high level MVA, but punishes those who are less productive. **B.** Maximal growth rate and MVA production by the strain equipped with product addiction system during simulated fermentation. **C.** Maximal growth rate and MVA production by the control strain equipped without product addiction system during simulated fermentation. MVA, mevalonic acid; *folP*, coding gene of dihydropteroate synthase (FolP); *glmM*, coding gene of phosphoglucosamine mutase (GlmM); atoB, acetoacetyl-CoA thiolase; HMGS, HMG-CoA synthase; tHMGR, truncated HMG-CoA reductase.

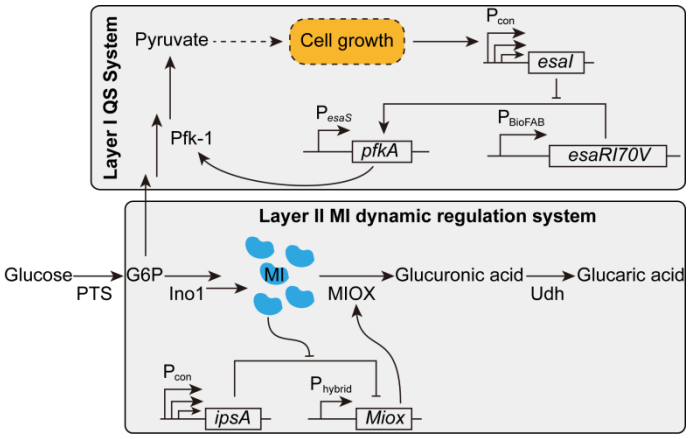

Figure 4. Layered dynamic regulation to improve glucaric acid production.

Two orthogonal, autonomous, and tunable dynamic systems are layered in the engineered cell. Layer I, pathway-independent quorum sensing (QS) system; layer II, pathway-dependent MI biosensor-based dynamic regulation system. PTS: phosphoenolpyruvate-dependent phosphotransferase system, responsible for the uptake and concomitant phosphorylation of glucose. G6P: glucose 6-phosphate. Ino1: *myo*-inositol-1-phosphate synthase (MIPS, from *Saccharomyces cerevisiae*), converting glucose-6-phosphate to *myo*-inositol-1-phosphate. MI: *myo*-inositol. MIOX: *myo*-inositol oxygenase. Udh: uronate dehydrogenase. *ipsA*: transcription factor IpsA coding gene. *Miox*: *myo*-inositol oxygenase MIOX coding gene. P_{con}: constitutive promoter. P_{hybrid}: engineered hybrid promoter that contained *E. coli* σ^{70} RNA polymerase recognition regions and IpsA binding site, permitting IpsA-mediated transcription. Pfk-1: phosphofructokinase-1. *pfkA*: Pfk-1 coding gene. *esaRI70V*: transcriptional regulator EsaRI70V coding gene. *esaI*: 3-oxohexanoylhomoserine lactone (AHL) synthase EsaI coding gene. P_{esaS}: *esaS* promoter that can be activated by EsaRI70V at the absent of AHL. P_{BioFAB}: a constitutive promoter from the BioFAB library.