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Development of biosensors and their application in metabolic engineering

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In a sustainable bioeconomy, many commodities and high value chemicals, including pharmaceuticals, will be manufactured using microbial cell factories from renewable feedstocks. These cell factories can be efficiently generated by constructing libraries of diversified genomes followed by screening for the desired phenotypes. However, methods available for microbial genome diversification far exceed our ability to screen and select for those variants with optimal performance. Genetically encoded biosensors have shown the potential to address this gap, given their ability to respond to small molecule binding and ease of implementation with high-throughput analysis. Here we describe recent progress in biosensor development and their applications in a metabolic engineering context. We also highlight examples of how biosensors can be integrated with synthetic circuits to exert feedback regulation on the metabolism for improved performance of cell factories.

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Introduction

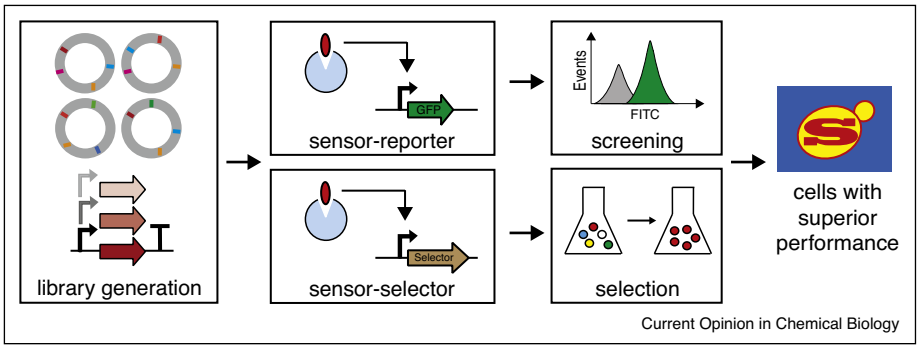
During the past two decades, metabolic engineering has emerged as an enabling technology for a sustainable bioeconomy [1]. It is envisioned that a significant fraction of fuels, commodity chemicals and pharmaceuticals will be produced from renewable feedstocks using microorganisms instead of relying exclusively on petroleum refining or extraction from plants [2]. To build microbial cell factories that can efficiently convert a feedstock into a desired product, metabolic engineers often adopt targeted and rational engineering of metabolic pathways [1,2]. On the other hand, non-targeted methods, such as directed evolution, have also proven effective in generating cell factories with improved performance (Figure 1) [3–7]. Moreover, with the continuous decrease in DNA synthesis costs and various efficient cloning and genome engineering methods [8–11], combinatorial procedures for library construction have become widely used in order to better understand metabolic pathways and their regulation [12,13]. Altogether, these versatile methods for generation of library diversity and the inherent evolvability of genetic material dramatically increase the potential of the host microorganism. However, the high capacity for diversity generation also imposes a need for efficient screening methods to select the individuals carrying the desired phenotype. Compared to diversity generation, versatile screening methods are lagging behind and are urgently needed to fully exploit the potential of large libraries. Conventional screening methods include color-based/spectrophotometry-based enzymatic assay or mass spectrometry (MS)-based analytics. Yet, all of these methods have limited throughput, unless the trait of interest can be coupled to an easy phenotypic output [14,15]. To circumvent this obstacle much effort has been made to develop genetically encoded biosensors that enable *in vivo* monitoring of cellular metabolism. When coupled to a reporter gene (e.g., GFP) or non-native selection (e.g., antibiotic resistance), biosensors offer the potential for high-throughput screening and selection of large diversified libraries using fluorescence-activated cell sorting (FACS) and cell survival, respectively.

This review will focus on recent progress in genetically encoded biosensors, specifically in microbial hosts. Here, examples from major categories of biosensors will be reviewed. As many biosensors are still in the 'proof-of-concept' stage, this review will also address aspects regarding their potential application in a metabolic engineering context to accelerate cell factory development. Finally, recent examples on integration of biosensors into genetic circuit regulation will be discussed.

Förster resonance energy transfer (FRET) sensors

Förster (or fluorescence) resonance energy transfer (FRET)-based sensors typically involve a pair of donor and acceptor fluorophores. A ligand-binding peptide is

Figure 1



Genetically encoded biosensors and their potential applications in high-throughput screening. Large libraries with genetic diversity can be generated by various means such as mutagenesis using error prone PCR or combinatorial *in vivo* assembly of DNA fragments. Sensor–reporter combinations allow high-throughput screening of cells using FACS to select for the desired trait. In addition, sensor–selector combinations can also be applied for the selection of mutant cells that exhibit superior performance.

sandwiched between the two fluorophores, so that when it is bound by the ligand of interest the peptide goes through a conformational change, causing a change in the proximity of donor and acceptor fluorophores and thereby a FRET change [16]. Owing to their sterical mode-of-action, FRET sensors do not require any other host cell components than the transcription and translation apparatus, making them a popular orthogonal screening tool [17,18]. For instance, the vast number of metabolite-binding protein scaffolds found in nature has served as a resource, from which platform FRET sensors for metabolite accumulations can be designed. In addition to the numerous examples of ligands including sugar phosphates [17], amino acids [18,19], carboxylic acids [20], cofactors [21], ions [22–24], FRET sensors can also be adopted to sense intracellular redox status [25] and other events that may be otherwise difficult to monitor (e.g., macromolecular crowding) [26].

Despite high orthogonality, temporal resolution, and ease of construction, FRET sensors are merely able to report the abundances of metabolites of interest without being able to exert downstream regulation in response to the signal. Also, the relatively low dynamic range observed between 'ON' and 'OFF' states may require time-consuming tuning of the 'bait' design according to the operational range of the input. For these reasons, FRET sensors have predominantly been applied in monitoring intracellular metabolite dynamics, rather than screening large cell factory libraries.

In the following sections we will cover current efforts on development and application of biosensors amenable for interaction with the host metabolism.

Transcription factor-based biosensors

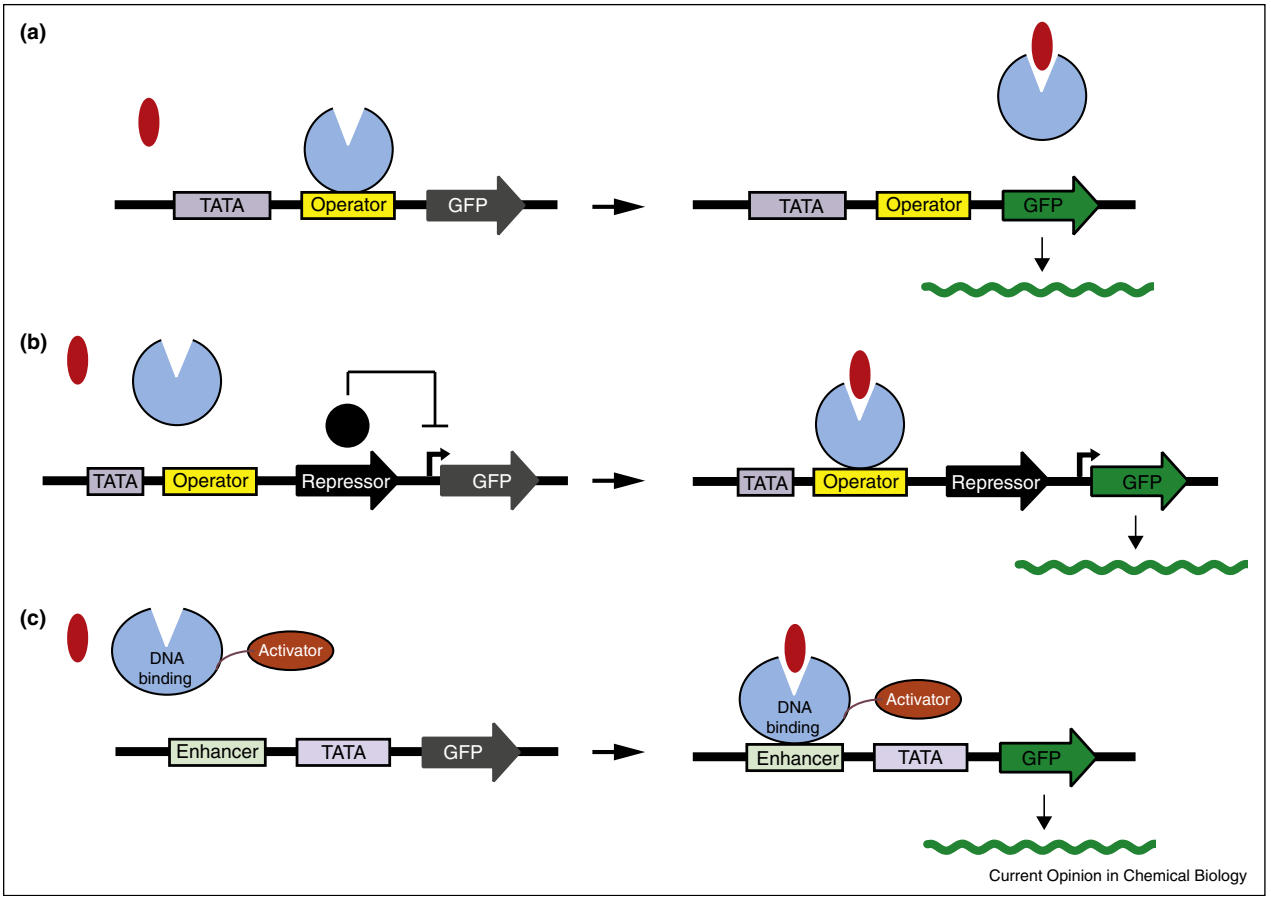
Transcription factors (TFs) are natural sensory proteins that have evolved to regulate gene expression in response to environmental changes or key intracellular signals that need tight control. Hence, it is not surprising that one straightforward approach to exploit TFs for high-throughput screening is to hack into the host transcription system and employ a synthetic or native condition-specific promoter to drive the expression of a reporter gene. For instance, Siedler *et al.* developed an NADPH/NADP$^+$ redox sensor in *Escherichia coli* using its native redox sensitive TF SoxR [27•]. Similarly, Knudsen *et al.* constructed a yeast NADH/NAD$^+$ redox sensor by expressing GFP under the control of *GPD2* promoter, which is known to be activated by an increased NADH/NAD$^+$ ratio [28]. Both redox sensors were further applied to screen and select enzyme variants that showed either improved enzyme activity or higher selectivity to a specific cofactor [27•,28].

Despite the simplicity in their design, a sensor–reporter system based on native TFs may suffer from poor orthogonality and background noise due to yet uncharacterized interactions between candidate TFs and operator sites in native promoters [29,30]. Furthermore, metabolic engineering applications often employ heterologous pathways to produce non-native chemicals, which may not be directly sensed by any native TFs. For biosensor design, these challenges can be tackled by introducing heterologous metabolite-binding TFs from organisms known to respond to such chemical cues. Here, transcriptional repressors from prokaryotes offer a vast toolkit of potentially orthogonal metabolite-binding TFs amenable for biosensor applications in eukaryotes. Indeed, early work has shown that prokaryotic transcriptional repressors can function in eukaryotes, though major differences exist between transcriptional regulation in eukaryotes and prokaryotes [31]. For instance, as in their native host, operator positioning at reporter promoters has proved instrumental for optimal sensor–reporter design when transferring repressors from prokaryote to eukaryote chassis [32•,33] (Figure 2a). In addition to those transcription

repressors that dissociate from operators upon ligand binding, another type of prokaryotic transcription repressor that repress reporter promoters upon ligand binding can also be engineered as orthogonal biosensors. In such cases, the reporter output would be negatively correlated with the concentration of the ligand, which can challenge the screening process (i.e., a higher risk of false positive selection). In such cases, the negative input–output correlation can be reversed using a two-layer NOT gate design, in which a first stimuli represses the action of a second repressor (Figure 2b) [34].

In addition to the inherent regulatory potential of TFs, the high degree of modularity also makes TFs a superb starting point for biosensor development. TFs contain at least a protein interaction domain to recruit transcriptional machinery, as well as a DNA binding domain (DBD), which often resides in proximity to the metabolite-binding domain (MBD). The high degree of modularity makes it possible to engineer specifically the MBD to improve its affinity and selectivity towards a new molecule [35]. Another way to take advantage of this modularity is by domain swapping to create hybrid TFs with novel properties. As an example, Moser *et al.* showed that a fusion of the activator domain of yeast Gal4 to the DNA binding and regulatory (i.e., sensory) domains (RD) of the bacterial TF Ada would respond to a methylating reagent, resulting in a hybrid activator that can also sense methylating reagent in yeast [36•] (Figure 2c). In another study, Umeyama *et al.* engineered a *S*-adenosylmethionine (SAM) biosensor in yeast by fusing the DBD and MBD of *E. coli* MetJ repressor to the AD of the B42 activator. The resulting hybrid activator, when bound by SAM, can bind to the operator (metO) inserted upstream the TATA box [37•]. This hybrid strategy can be generally applied to 'eukaryotize' those prokaryotic TFs that

Figure 2



General strategies for harnessing TFs as biosensors. **(a)** One type of prokaryotic repressors that can bind to its operator in the absence of its ligand and dissociate from DNA when the ligand becomes available. This mode of action can be exploited to sense the level of the ligand, using this repressor to control GFP expression; **(b)** in another scenario, the repressor binds to its operator when the ligand is present. To obtain a positive correlation between ligand availability and the reporter level, a second repressor could be expressed under the first repressor and can control a GFP; **(c)** a hybrid transcriptional activator can be constructed by fusing the activator domain of a eukaryotic TF to the DNA/inducer-binding domains of a prokaryotic TF, resulting a new TF that respond to the inducer of prokaryotic TF.

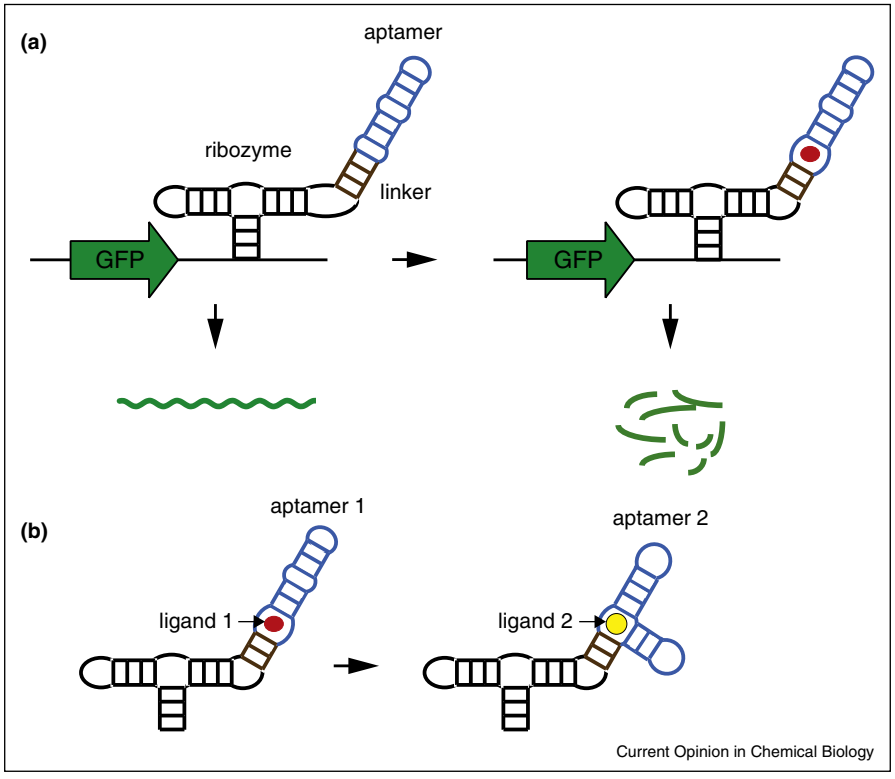
are responsive to a metabolite but not functional in eukaryotes.

Riboswitches

Riboswitches are a third class of biosensors. A riboswitch is the regulatory domain of an mRNA that can selectively bind to a ligand and consequently change its own structure, thereby regulating transcription or translation of its encoded protein [38]. Although many questions regarding their origins and functions are still to be answered, much effort has already been made to harness riboswitches as small molecule biosensors [39•]. Compared to TF-based sensor–reporter systems, riboswitches offer faster responses since the RNA has already been transcribed and therefore is readily available for effector binding. Furthermore, riboswitches do not rely on protein–protein or protein–metabolite interactions. This allows for more targeted engineering of the aptamers (the ligand binding domain) [40] and the expression platforms [41,42]. Accordingly, methods have been developed to create synthetic aptamer libraries, which can be *in vitro* selected towards a ligand of choice [43]. In two recent studies in *E. coli*, Pablo *et al.* demonstrated that expression platforms from existing riboswitches can be engineered using simple design rules to host natural or synthetic aptamers to create novel riboswitches [44•,45•]. This '*mix-and-match*' approach greatly expands the collection of new synthetic riboswitches with improved performance [46]. Computational methods have also been developed for *de novo* design of a synthetic riboswitch that regulates transcription termination [47].

In the last decade, riboswitches have been extensively engineered in bacterial systems, which were comprehensively reviewed in several papers [39•,48,49]. Compared to the efforts reported in bacteria, engineering of yeast riboswitches has lagged behind. This is probably because the vast majority of riboswitches are discovered in bacteria and non-functional in yeast due to distinct differences in transcription and translation between prokaryotes and eukaryotes. One strategy that can be relatively easily implemented in yeast is to utilize ribozyme-based switches, which upon ligand binding controls their self-cleavage activity and therefore translation [47] (Figure 3a). Michener *et al.* employed a theophylline-responsive ribozyme to control the expression of GFP, which was used to screen an evolved caffeine demethylase library in yeast [50•]. Following the same principle,

Figure 3



Engineering of ribozyme-based RNA switches in *S. cerevisiae*. **(a)** An aptamer that can specifically bind to the ligand of interest is linked to a ribozyme, resulting in an aptazyme that cleaves itself upon ligand binding. This leads to the instability of mRNA therefore inhibits the expression of the reporter protein (GFP); **(b)** a general strategy for creating novel aptazymes in yeast is by swapping the aptamer domain, which can be created for a specific ligand using systematic evolution of ligands by exponential enrichment (SELEX) [53].

Klauser *et al.* developed a ribozyme-based neomycin switch in *Saccharomyces cerevisiae* [51••]. Here, they first attach a synthetic neomycin aptamer [52] to the catalytic core of the type III *Schistosoma mansoni* hammerhead ribozyme (HHR), resulting in synthetic neomycin-dependent ribozymes, which were then subjected to a delicately designed *in vivo* selection method [51••] (Figure 3b). The best resulting riboswitch exhibited a 25-fold inhibition of gene expression upon neomycin binding, representing the highest dynamic range so far reported. Given the high degree of modularity in riboswitches, it can also be a general strategy to combine *in vitro*-selected synthetic aptamer to the catalytic core of ribozyme such as HHR to create novel yeast synthetic riboswitches.

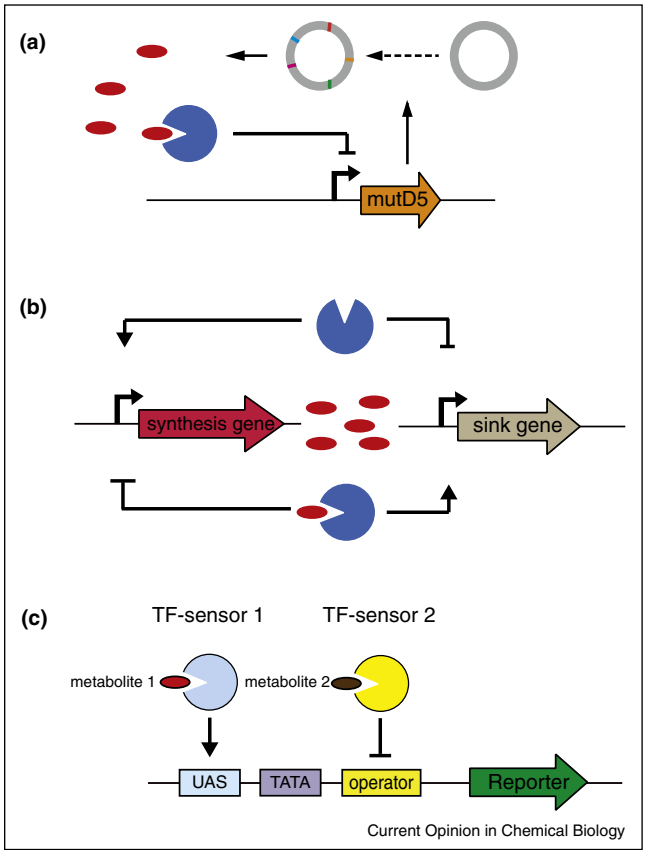
Integration of biosensors into synthetic circuits

As an emerging research discipline, synthetic biology plays a synergistic role in advancing metabolic engineering by providing well-characterized, standardized and robust parts, which can minimize the need for extensive tuning each part during construction of cell factories [54,55]. Furthermore, synthetic biology can facilitate metabolic engineering with the design of sophisticated genetic circuits that, when coupled to a biosensor, can carry out various biological functions for a more robust host tailored for advanced applications [56].

One type of circuit engages a biosensor to regulate a marker gene to facilitate genetic selection. Using such a sensor–selector system, Yang *et al.* demonstrated the selection of lysine high-producing strains using an 'OFF' riboswitch from *E. coli lysC* to repress the expression of a suicide selector tetracycline/H^+ antiporter (encoded by *tetA*), which confers toxicity by importing toxic metal ions (Ni^{2+}) to the cell [57••]. Dietrich *et al.* took another strategy by harnessing the σ^{54}-factor BmoR and the corresponding promoter (P_{BMO}) as an *n*-butanol biosensor in *E. coli* [58•]. Here, TetA-GFP fusion protein was expressed under this TF-promoter pair for simultaneous reporting of TetA expression and tetracycline resistance, which was successfully implemented for synthetic selection of the *n*-butanol production phenotype. In yeast, Lee *et al.* recently engineered a synthetic suicide switch by coupling the yeast cytosine deaminase (*FCY1*) to *glmS*, a glucosamine 6-phosphate (GlcN6P)-responsive ribozyme [59•]. Supplementation of fluorocytosine confers toxicity, which can only be alleviated by inhibiting *FCY1* expression upon GlcN6P binding, thereby providing a screening strategy for the selection of a GlcN6P high-producing mutant strain that can be used for bio-based production.

In addition to the genetic selectors mentioned above, biosensors can also be coupled to an actuator for the dynamic regulation of a cellular component (e.g., enzyme, pathway, etc.) according to the input signal. Chou *et al.* developed a system termed as *Feedback-Regulated Evolution of Phenotype* (FREP), where the actuator is a mutator (encoded by *mutD5* in *E. coli*) controlled by a TF-based biosensor (Figure 4a) [60••]. When the sensor was the *E. coli* TF-promoter pair TyrR-P_{aroF}, the authors showed a 5-fold increase in tyrosine production. A hybrid TF comprising the MBD of isopentenyl diphosphate (IPP) isomerase and the AD and DBD from *E. coli* TF AraC was constructed and applied to control *mutD5* expression, which yielded a 3-fold improvement in lycopene production. This method represents a combination of continuous *in vivo* evolution and a synthetic sensor–actuator circuit that facilitate screening. With the successful development of an orthogonal error-prone replication

Figure 4



Integration of biosensors into genetic circuits. **(a)** A sensor–actuator system can be implemented to mediate the continuous *in vivo* evolution of desired phenotype. The evolution only stops after the evolution goal is met; **(b)** a sensor–actuator system has proved effective in the dynamic regulation of FAs or FAEE biosynthesis in *E. coli*. This feedback regulation can be beneficial as it essentially balances the fluxes between growth and production of target chemicals; **(c)** logic AND gates can be employed to integrate input signals from multiple biosensors, which have potential usage, for example, in the conditional expression of metabolic enzymes to coordinate with nutritional availability in industrial scale fermentation.

system in yeast, FREP may not be too far from implementation for advancing yeast metabolic engineering [61•]

In addition to sensor–selector systems and continuous evolution studies, dynamic feedback regulation of a heterologous pathway can be used to optimize metabolic fluxes by minimizing unnecessary protein expression and the accumulation of toxic intermediates. Zhang *et al.* designed a dynamic sensor (fatty acid acyl-CoA responsive TF FadR)–regulator system to regulate the production of ethanol, which is both a precursor for fatty acid ethyl esters (FAEE) and a toxic byproduct (Figure 4b) [62••]. In a similar approach, Xu *et al.* built a biosensor (malonyl-CoA responsive TF *FapR*)–regulator circuit, in which acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) are dynamically controlled by malonyl-CoA level (Figure 4b) [63••]. These strategies are generally useful for engineering microbial production of bulk chemicals that are derived from central precursors (e.g., acetyl-CoA). A tight regulation of these biosynthetic pathways can avoid over-draining those key precursors by fine-tuning the balance between cell growth and production.

Finally, synthetic logic gates can be useful for integrating multiple input signals from different biosensors. As an example, a logic AND gate can be implemented to output a signal only when both inputs are positive. For riboswitch-based sensors, these logic gates can be built using ribozymes that contain multiple aptamers for different metabolites, each serving as a sensor for one specific signal [64]. In the case of TF-based sensors, Teo *et al.* illustrated recently the construction of such a logic AND gate comprising the FadR and a synthetic promoter containing *GAL1* core promoter and the upstream enhancer element (UEE) from either a copper inducible *CUP1* promoter or phosphate repressible *PHO5* promoter (Figure 4c) [32•]. In another aforementioned study of a hybrid TF-based sensor for SAM, the author also used doxycycline (DOX)-responsive TetR-TetO, which was inserted downstream of TATA, to integrate a second signal to tune the expression of the selection marker [37•]. For yet another hybrid TF, Farzadfard *et al.* designed conditional expression (presence of galactose) of a nuclease-deficient Cas9 variant guided towards its regulatory target only in the presence of tetracycline, which served to derepress TetR-controlled gRNA expression in yeast [65]. These examples illustrate how biosensors can be implemented in synthetic logic gates that can be used for conditional expression of enzymes in industrial fermentation, depending on the growth phase or nutritional availability.

Conclusions

Environment concerns and lack of sustainability of petroleum-derived products are steadily driving the development of microbial cell factories for the production of chemicals. Metabolic engineers have made tremendous progress in optimizing the cell metabolism for efficient conversion of feedstock to target compounds. With continuous *in vivo* evolution [60••,61•] and improved methods for high-efficiency genome engineering [9–11], the bottleneck of successful biological transformations often becomes the screening capacity. As reviewed here, biosensors have shown great potential in both high-throughput screening of large diversified libraries and implementation of synthetic circuits for dynamic control of cell metabolism [62••,63••]. In order to go from 'proof-of-concept' to industrial scale applications, efforts need to be prioritized into development of novel biosensors and characterization of their performance at industrial scales. Future challenges also include how to integrate these biosensors with other controllable genetic devices to build unprecedented microbial cell factories, where synthetic biology can serve as an excellent platform.

Conflicts of interest

The authors declare no conflicts of interest

Acknowledgement

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- •• of outstanding interest

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