



Wiring cell growth to product formation

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Microbial cell factories offer new and sustainable production routes for high-value chemicals. However, identification of high producers within a library of clones remains a challenge. When product formation is coupled to growth, millions of metabolic variants can be effectively interrogated by growth selection, dramatically increasing the throughput of strain evaluation. While growth-coupled selections for cell factories have a long history of success based on metabolite auxotrophies and toxic antimetabolites, such methods are generally restricted to molecules native to their host metabolism. New synthetic biology tools offer the opportunity to rewire cellular metabolism to depend on specific and non-native products for growth.

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Introduction

Toxic waste-products and dependency on petrochemicals are notably problematic in the chemical synthesis industry. A forward-thinking solution is replacing manufacture of materials, medicines, and biochemicals by engineered microbial processes. Despite promising benefits and a growing interest, widespread industrial implementation of biotechnology has been constrained by long development timelines and process economics due to the challenges of strain engineering [1].

The expansion of the DNA modification toolbox allows genetic editing in a targeted, rational manner, and this has generated a profusion of engineering strategies based on innovation in DNA synthesis and genome editing [2]. Yet the technology to investigate the resulting clones is often

based on low-throughput, traditional analytics which cannot address the number of clones present in such diverse libraries.

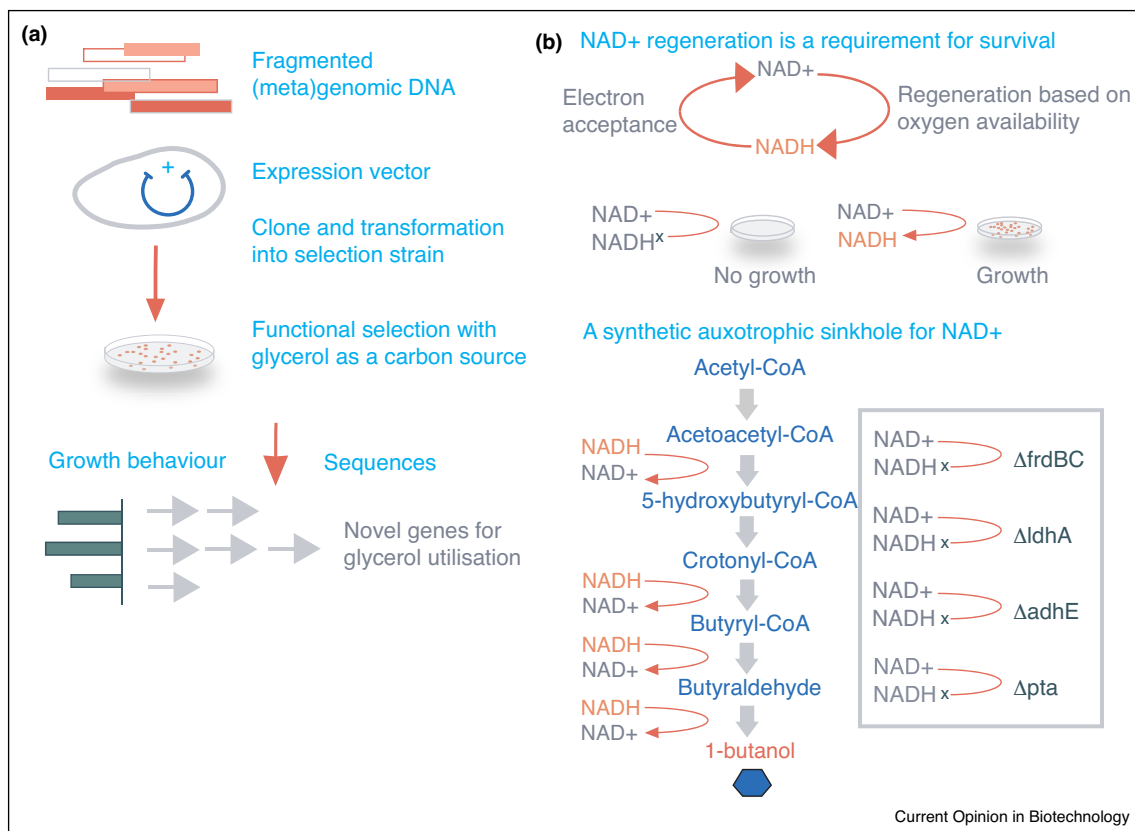
Biological reporter systems that link product formation to an immediately detectable output represent a potential solution to accelerate laborious screening protocols using either fluorescence-activated cell sorting [3^{*}] or microfluidic droplet based sorting [4]. However, the reporter systems with the largest throughput are those that allow for growth selection by wiring product formation to cell growth [5^{**},6]. With a focus mainly on *Escherichia coli*, we describe recent work on applications of conventional selection strategies, review synthetic selection systems, and illustrate the difficulties that arise from the construction and use of such approaches when deployed for industrial purposes.

Expanding on conventional selection systems

Conventional selection systems have been employed in industrial biotechnology since the 1970s and often rely on auxotrophic knock-outs and inhibitory molecules to construct dependencies on the target molecule. One implementation of gene deletions is the discovery of novel sequences for glycerol utilization using functional metagenomic selections [7,8] (Figure 1a).

Auxotrophic strains are a powerful tool for selection, but not all molecules of interest are amenable for this type of growth-coupling. Hence, the construction of non-native auxotrophies has been pursued. For example, 1-butanol is an important fermentation product from *Clostridia* spp. The native pathway is strongly CoA-dependent and this co-factor demand has challenged optimization or heterologous transfer. Particularly in *E. coli*, the metabolic state of high CoA is only available during anaerobic recycling of NADH via mixed-acid fermentation. In several studies multiple knockouts of electron acceptors leave 1-butanol as the only 'NADH outlet' for redox balance under anaerobic growth [10^{**},11]. Without high flux through the 1-butanol pathway, lack of NAD⁺ regeneration leads to growth arrest. The redox dependency enabled the selection of mutants with increased activity of CoA reductase from error-prone PCR mutagenized libraries and fermentation titers for butanol were improved to 88% of the theoretical yield, reaching 30 g/L. The authors note the suitability of this anaerobic growth rescue approach for other NADH-dependent products, including lactate, alanine, or succinate.

Figure 1



Utilizing auxotrophy for growth-dependent selection.

(a) Functional metagenomics selections can be utilized in metabolic engineering. Here, DNA fragments of a metagenomic library are selected for improved capacity to utilize glycerol as a carbon source [9,8].

(b) The NAD⁺/NADH redox requirement of *E. coli* is utilized to build a strain dependent on 1-butanol product formation. As other mixed-fermentation reactions are knocked out (grey box), NAD⁺ can only be regenerated by the 1-butanol pathway [10**].

The success of an industrial process can hinge on the choice of a suitable production strain and overlap of target molecules with metabolic dependency should be considered. Computational resources that identify relevant gene knock-outs are useful tools [12*,13]. Predictive genome-scale modelling methods, such as constraint-based reconstruction and analysis (COBRA) methods, can probe further options for engineering a synthetic metabolic link for compounds of interest such as 2-oxoglutarate, succinate, or limonene [14–17]. These tools are introducing powerful computational resources for a technology traditionally based in the lab.

Another selection approach to strain engineering relies on antimetabolites, which are metabolite analogs that inhibit growth [18]. This can be due to incorrect substrate recognition leading to enzymatic inhibition or disruption of pathway regulation. At the correct concentration, antimetabolites force the cell to elevate enzyme concentrations or small-molecule products to overcome the inhibition burden. For example, three structural antimetabolites enabled

selection of strain variants for aspartic acid production with up to fourfold increase of fitness in heterologous populations [19]. In another study, riboflavin production was increased from below 35 mg/L to 680 mg/L in *Candida famata* by the use of structural analogs of riboflavin in combination with a color-based screen for the vitamin [20**]. Antimetabolites can also be used to select for increased tolerance to toxic pathway intermediates [21,22].

A further type of selection can be imposed by light-excitable quantum cadmium telluride dots. The dots generate ROS-stress with the assumption that increased tolerance to superoxide is correlated with strong NADPH metabolism, key for industrial molecules. A loss-of-function mutation in *hdfR* was identified using this selection and exhibited a twofold increase in titers for 3-hydroxypropionic acid, a NADPH-limited pathway [23].

Conventional selections are powerful tools for metabolic engineering and expanding them with synthetic biology

tools is pushing their versatility even further. Muconic acid production in *Saccharomyces cerevisiae* was improved threefold by combining a biosensor conferring geneticin resistance with ALE against 4-fluorophenylalanine, a competitive inhibitor of aromatic amino acids. The evolved strains gave titers of 2.1 g/L [21]. Next-generation sequencing gives insight into population behavior [19] and selections can be combined with rational engineering [19,20**].

Nevertheless, important limitations remain for both anti-metabolite and auxotrophic selections. Auxotrophic demands exist only for essential metabolites, and this molecular requirement must lie in the range of titers relevant to industrial processes. Unfortunately, this is rarely the case and accordingly selection options arising from auxotrophic or inhibitory elements can be limited.

Synthetic coupling of growth to product formation

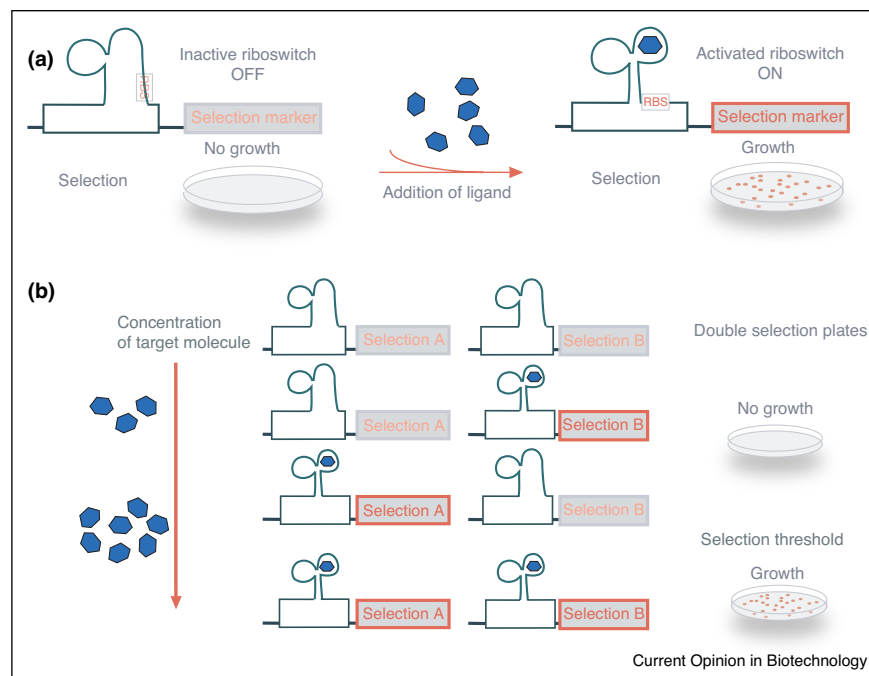
Synthetic growth-coupled systems have the same aim as conventional strain engineering: identification of high producers from a pool of clones. Starting from ligand-responsive switches, gene networks are rewired to monitor the presence

of target molecules. Gene switches can be based on riboswitches (Figure 2) or transcription factors (TFs) (Figure 3), which are then linked to selectable marker genes to generate a synthetic or non-natural coupling of growth to product formation.

A seminal selection system utilized the specific response of the NahR TF to benzoic acids but not the corresponding aldehydes and coupled it to tetracycline resistance [24]. The selection identified increased enzymatic activity of *xyfC*, a benzaldehyde dehydrogenase from *Pseudomonas putida*. A separate system, focused on lysine production, repurposed a riboswitch upstream of *lysC* to build a 'Riboselector' such that the presence of lysine reduces toxic expression of *tetA* [25]. In four rounds, a proof-of-concept plasmid expressing varying promoter strengths for *ppc*, a key node for the lysine pathway, was enriched. In the same work, a tryptophan-specific Riboselector was generated from an aptamer functioning as an ON switch.

A selection system for thiamine, capable of single cell selection, was developed based on the natural ThiM19

Figure 2

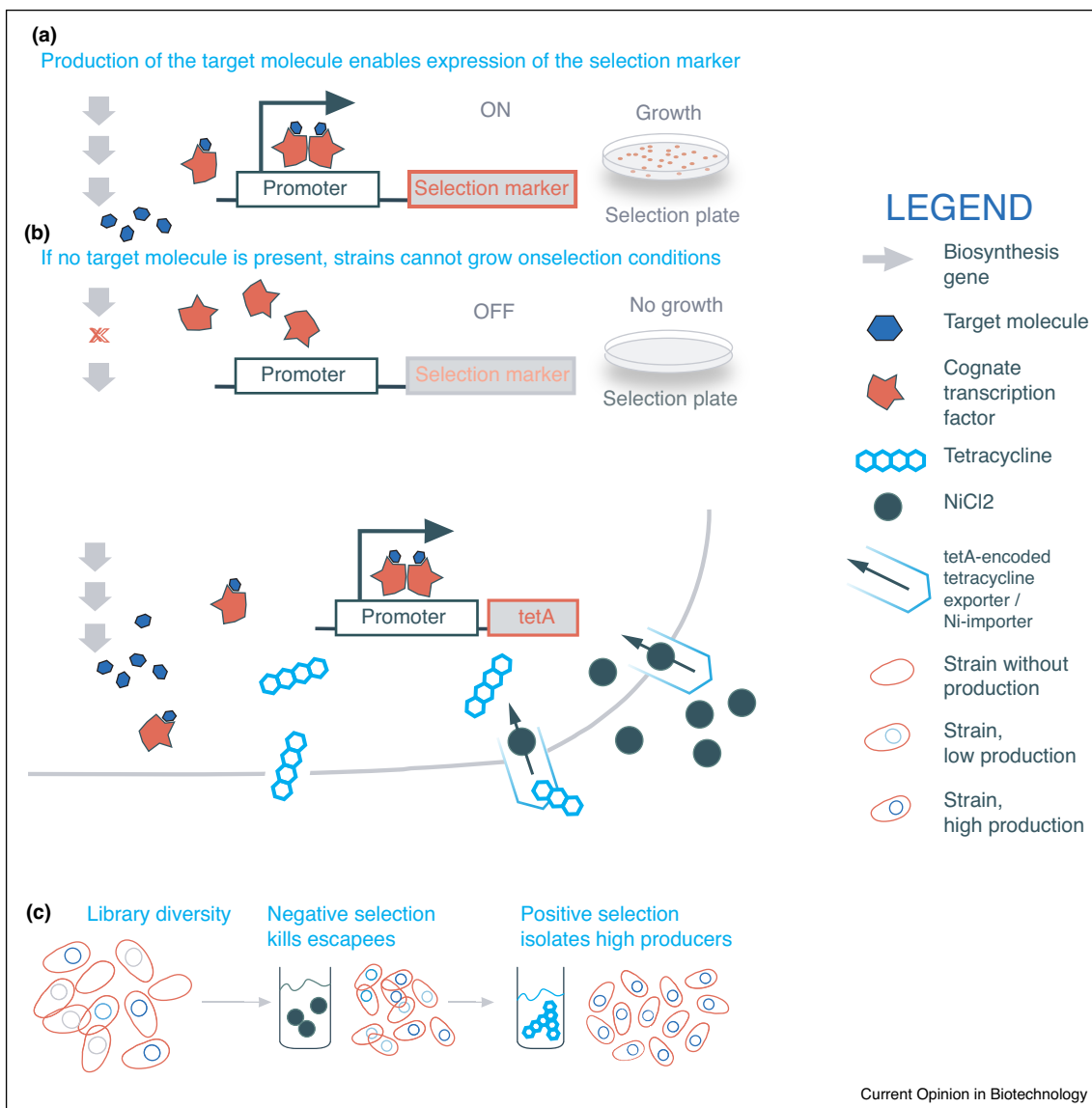


Riboswitch-based selection.

(a) A riboswitch is a regulatory element that acts at the level of RNA. Addition of the molecule of interest facilitates a structural change of the transcript, which can uncover (in this example) an RBS binding site. Shown (left) is a riboswitch without ligand in its OFF state so that the selection marker is not active (light grey). The addition of ligand allows expression of the selection marker, switching the selection to the ON state (dark orange) and allowing growth on selection plates.

(b) In order to generate a robust selection platform that avoids a high rate of false positives, Genee et al. [6] place two separate antibiotic resistance cassettes under control of independent thiamine pyrophosphate riboswitches, shown to control Selection A and Selection B. When no product is present, both riboswitches are inactive. In the case of point mutations, a single riboswitch would lose its selection pressure and allow growth on selection plates. Instead, the set-up confers resistance to both selection antibiotics only for i) high product concentrations and ii) no loss of selection to activate both riboswitches to the ON state.

Figure 3



Transcription-factor based selection.

(a) Biosynthesis of a target molecule is coupled to a cognate transcription factor which often dimerizes to activate or repress a selection marker. Shown is a positive selection marker where the presence of the target molecule enables expression of an antibiotic resistance marker, allowing only producers to grow on a selection plate.

(b) Negative selection strategies arise from markers with dual effects on the cell. For example, gene *tetA* provides resistance to tetracycline but makes the host cell susceptible to NiCl₂ toxicity. Negative selection functions ensure that selection 'winners' have not bypassed the selection pressure.

(c) A workflow for a joint selection system with a positive and negative round is shown. From a diverse library, high producers are isolated using a round of positive selection. Before screening, a negative selection step ensures that the selection pressure has been maintained to rule out false positives.

riboswitch [6,26,27]. In the presence of thiamine pyrophosphate, the repurposed ThiM19 riboswitch allows translation initiation of an otherwise repressed gene. In the study, the switch was coupled to an antibiotic resistance cassette, thereby generating a synthetic selection strain growth-dependent on thiamine pyrophosphate.

To apprehend selection escapees, the system was expanded to a second thiamine pyrophosphate riboswitch with a different antibiotic resistance gene, which reduced the rate of selection evasion by 1000-fold (Figure 2b). The established selection system was expanded for xanthine alkaloid selection [6] and applied

to metagenomic gene discovery and transport engineering [28]; demonstrating its versatility and selection power.

As with the NahR example, further synthetic selections often utilize TF-based metabolite sensing to conditionally express antibiotic resistance cassettes [29,30^{••},31,32]. A heterologous activator and promoter from *Thauera* sp. conditionally expressed the antibiotic resistance cassette *tetA* in the presence of 1-butanol. This system was used to screen $\Delta adhE$ strains for plasmid-encoded RBS libraries of heterologous *kivD* and *ADH6* from yeast, which identified a strain with increased activity of 35% [30^{••}]. Two additional systems were built to showcase the utility of conditional *tetA* expression. Firstly, the native *E. coli* succinate pathway is regulated by the two-component system, DcuR/DcuS, which was coupled to *tetA* expression. Secondly, a system for adipate was built from *P. putida* TF PcaR, also linked to *tetA* [30^{••}].

Nevertheless, evolutionary escapees can overwhelm selection systems [6,31]. A ‘toggled’ approach reduced this issue by alternating between positive and negative selection rounds (Figure 3c). In addition to enriching for high producers, the selection kills the fraction of non-producers that have mutated to evade the selection system [33]. Using both *tolC* and kanamycin resistance under a single conditional module can generate a kanamycin-resistant strain susceptible to colicin-E1 [31]. Selection winners can be tested for false positivity by colicin-E1 susceptibility. Toggled selection was used to identify a 36-fold improved naringenin producer from genome-engineered libraries using *tolC* under regulation of TtgR. In a second example, *tolC* regulated by CdaR increased glucaric acid production 22-fold [31].

Synthetic selections have also been applied to increase protein expression and export. To avoid extraction protocols, secretion is advantageous for recombinant protein production. A common approach is to fuse the protein of interest to naturally secreted substances such as YebF or OsmY, but this suffers from low yields [34]. Coupling of YebF to BLiP, a β -lactamase inhibitor protein, conveys increased resistance against β -lactam antibiotics. This approach was used to find mutants with improved extracellular accumulation of desired proteins in libraries with 10^{12} members [34].

Challenges of developing synthetic selection systems

At the heart of many selection systems are genetic switches harvested from nature. Yet, the construction of novel selection systems from an identified switch is challenging. Nevertheless, recent developments point toward efficient design strategies that could be applied to expand the repertoire of selections.

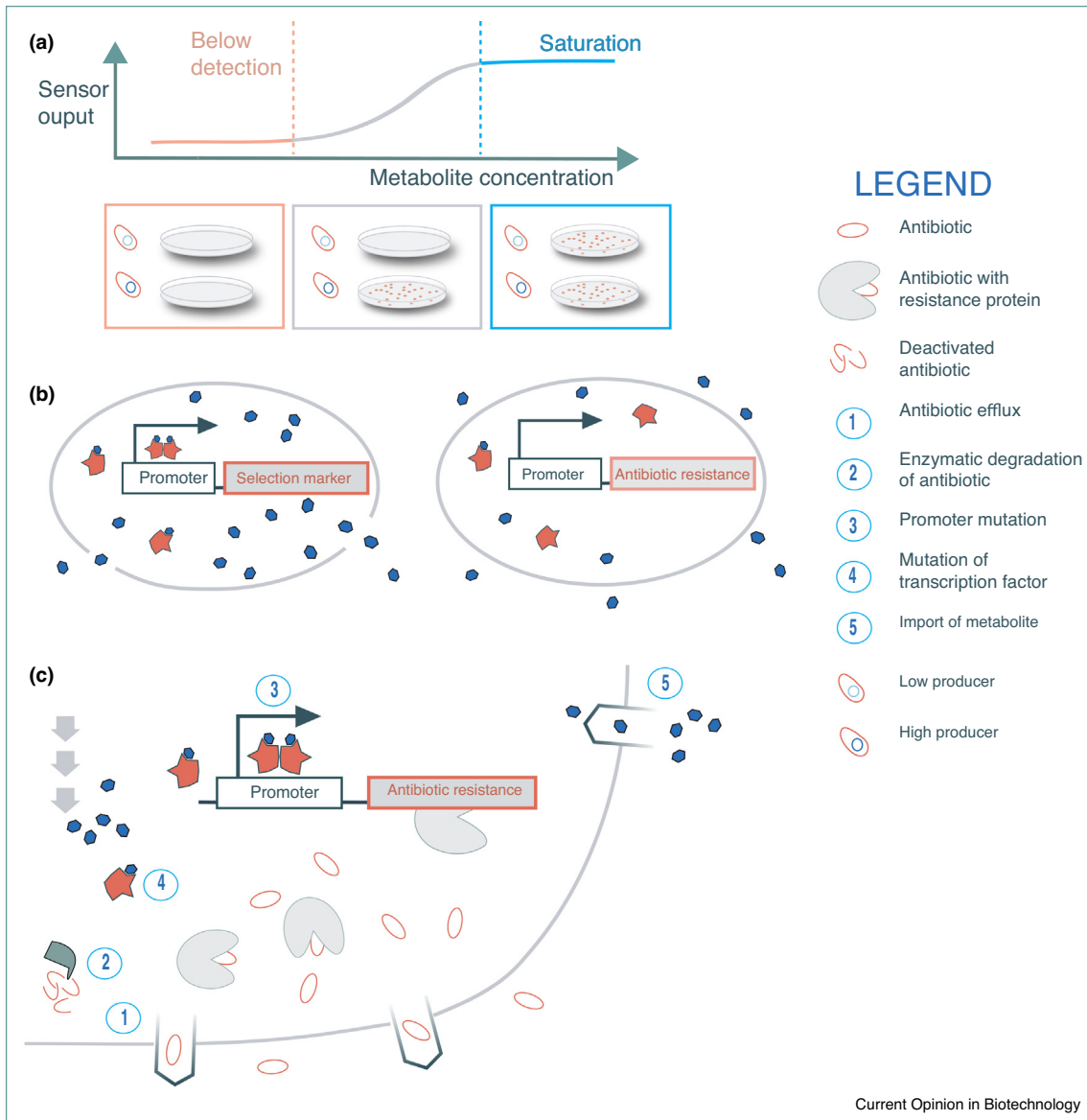
Despite the prevalence of regulatory elements across diverse genomes, the identification or construction of

robust metabolic sensors can be laborious for either protein or RNA-based sensors [35]. SIGEX (substrate-induced gene-expression screening), which places a promoterless reporter gene on either side of a randomized metagenomic region in the presence of the desired target, is a powerful tool. If a transcription-factor-like sequence is present, the reporter gene will be expressed [36]. The identification of specific aptamers using SELEX (systematic evolution of ligands by exponential enrichment) and the rational design of riboswitches are also not trivial [37,38], as the relationship between sequence and function of RNA is not readily discerned [39]. Modelling RNA sequence to function may assist in the future construction of riboswitch-based selection systems by tapping into the power of algorithmic approaches [40,41]. An approach called ‘term-seq’ identifies natural riboregulators in genome sequences via early termination events. This methodology is independent of evolutionary conservation and can find riboswitches that comparative genomics would not [37,38].

After identification, biosensors can still behave unexpectedly in the production host. Insufficient specificity can reduce the applicability of a biosensor. For example, the adipate TF-sensor also responded to pimelate (3et0). Nevertheless, non-specific biosensors can be powerful for some applications. One study used a geneticin-expressing proxy biosensor, which responded to a pathway intermediate instead of the target molecule, to identify overproduction strains [21]. Further, co-cultivation strategies can overcome the difficulties of having selection and production in a single strain. A ultra high-throughput application of fluorescence markers was used to screen multiplexed libraries and identify a *Bacillus subtilis* producer for riboflavin via alginate co-encapsulation with an *E. coli* sensor strain [42]. Proxy biosensors and co-cultivation may offer solutions when appropriate TFs or riboswitches are not available.

A separate issue concerns biosensor range, that is, metabolite concentrations which lead to discernable signal changes (Figure 4a). Dynamic ranges are rarely appropriate for continuous selection systems with increasing titers. A fluorescent biosensor for L-valine production in *Corynebacterium glutamicum* enabled increased titers by 25% for 5 rounds of FACS sorting before the upper detection limit had been reached [43]. The 1-butanol sensor described above [30^{••}] could not be used above 25 mM metabolite concentration. The glucaric acid system [31] also reports the range’s limitations. A proffered solution is transport regulation to direct the rate of molecule uptake and thereby the dynamic sensor range [31] (Figure 4b). A further strategy is the construction of molecular buffer systems. In this work [44] a TF is split into two functional domains, DNA-binding and activation. An excess of DNA binding domain mimics chemical pH buffer systems to generate a robust signal and protection against promoter leakiness.

Figure 4



Tuning selection systems and limiting evolutionary escape.

(a) Tuning of a selection system.

The graph indicates the output of a selection marker based on metabolite concentration. Two saturation points are indicated; a lower threshold where decrease in production does not lead to decrease of output (orange dotted line) and an upper threshold where increase in production does not lead to increase in output (blue dotted line). The colored boxes below show the effect of high or low production at each of the three phases, emphasizing the importance of biosensor range to get a screenable output correlated with product formation.

(b) As in Raman *et al.* [31], engineering of transporters can shift the functional range of a selection system. Two sensors are shown, with (left) or without (right) active import, which changes the conditional expression of the selection marker even though the extracellular metabolite concentrations remain the same.

(c) Loss of selection via evolutionary escapees.

Overview of a cell with a generic growth-coupled selection system: a transcription factor binds to the molecule of interest, dimerizes, and activates the expression of an antibiotic resistance cassette. Numberings 1–5 illustrate potential mechanisms of selection escapee. These mechanisms would allow the cell to survive an antibiotic challenge even if it did not produce the molecule of interest.

Finally, a major obstacle is the intrinsic response of biological systems to overcome selective pressure [45,46]. A single point mutation in promoter regions can confer loss of selection, which provides the escapee with unrivalled growth advantage compared to the remaining population (Figure 4c). The thiamine riboswitch gave a false positive rate of 10E-3 for an early, unoptimized selection construct [6]. In nature, genetic networks often display redundancy so that their function is robust to mutagenic effects. Gene duplication is a strong opposition to genetic drift, and this strategy can be utilized to reduce selection escape. Redundancy within selections can consist of two antibiotic resistance cassettes under one regulator [6] or single genes with two controllable outputs [33]. Raman *et al.* [31] demonstrate the versatility of selection doubling by expanding ten different biosensors in a modular way. The concept of redundancy is a powerful addition to the toolkit of selection systems.

Conclusion

Within industrial biotechnology, the need for robust, modular, and usable selection systems arise with the ability to manipulate, design, and multiplex the DNA code. Selection strategies of conventional strain engineering can identify genotypes with higher production capacity and are amenable for expansion with modern techniques. The further development of synthetic circuits to wire growth to product formation allows for the characterization of millions of genetic variants, with particular applications to generate sustainable cell factories for a greener chemical industry. Nevertheless, the success of synthetic selection systems will rely on overcoming the challenges of their construction and application: identification of relevant conditional modules, solving issues of specificity and range of *in vivo* biosensors, and addressing the false positives and negatives that arise from loss of selection.

Conflict of interest statement

Nothing declared.

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