Opinion

Synthetic Evolution of Metabolic Productivity Using Biosensors

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Synthetic biology has progressed to the point where genes that encode whole metabolic pathways and even genomes can be manufactured and brought to life. This impressive ability to synthesise and assemble DNA is not yet matched by an ability to predictively engineer biology. These difficulties exist because biological systems are often overwhelmingly complex, having evolved to facilitate growth and survival rather than specific engineering objectives such as the optimisation of biochemical production. A promising and revolutionary solution to this problem is to harness the process of evolution to create microbial strains with desired properties. The tools of systems biology can then be applied to understand the principles of biological design, bringing synthetic biology closer to becoming a predictive engineering discipline.

Design in Synthetic Biology Is Limited by Biological Understanding

Synthetic biologists currently possess an unprecedented capacity to construct large DNA sequences. Products of this construction process range from individual genes and promoters through to entire metabolic pathways and microbial genomes [1,2]. Although it is now possible to assemble synthetic DNA sequences of virtually any size, there is still a limited capacity to design biological systems that deviate significantly from their naturally occurring counterparts. This difficulty exists due to the complexity of biological systems and our incomplete understanding of genotype to phenotype relationships. One promising mechanism to circumvent these limitations in synthetic biology is to use the process of evolution to achieve engineering objectives. Evolutionary trajectories and their genetic basis can then be documented using the various omics tools of systems biology. In theory, this process can be used to learn new principles for the rational design of biological systems. This “reverse engineering” is beginning to be implemented in microorganisms for the production of valuable biofuels, chemicals, pharmaceuticals, and flavours and fragrances, where the complexity of biological systems is a constant barrier to the success of metabolic engineering efforts.

Current methods for overproducing target biomolecules involve the overexpression of relevant metabolic pathway genes, the elimination of enzymes that compete for carbon, and the balancing of ATP and reducing power (NADH and NADPH) [3]. High-profile successes in the field include the commercial production of the antimalarial compound artemisinin in the yeast Saccharomyces cerevisiae [4] and the production of the important industrial polymer 1,3-propanediol in Escherichia coli [5–7]. Developing microorganisms into “cellular factories” that produce a desired, non-native product at commercial yields (see Glossary) usually requires many millions of dollars, hundreds of person-years, and highly diverse expertise. This is because progress occurs as part of an iterative and time-consuming design/build/test cycle that is akin to a classical trial and error method. Although there are exceptions to this situation, such as the rational engineering of E. coli for the production of 1,4-butanediol [8], our

Trends

Synthetic biological systems can range in size and complexity from metabolic pathways to entire genomes.

Our capacity to assemble DNA sequences is not matched by an ability to predictively engineer novel biological functions because of the overwhelming complexity of biological systems.

Adaptive laboratory evolution (ALE) allows systems-biology approaches to be used to discover the genetic and physiological basis of evolved phenotypes, thereby informing rational design.

If ALE could be applied to evolve microbes for the production of target metabolites, then many of the bottlenecks that currently limit rational engineering in synthetic biology could be overcome.

Metabolite biosensors connect the intracellular concentration of a target molecule to a survival output. Genetically diverse populations can then be screened for superior producers that have novel genomic architectures.

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incomplete understanding of biology still limits the speed at which metabolic engineering can proceed.

The Evolution of Metabolism
All rational metabolic engineering efforts are fundamentally limited by the fact that a given organism has a genome that has evolved to execute survival and proliferation functions. From the first chemical information systems in the early biosphere to today’s modern genomes of extraordinary complexity, life has been governed by the imperatives of replication and survival [9]. The very best metabolic engineering efforts have rendered target chemical production the primary byproduct of normal growth-based metabolism. The ultimate goal of metabolic engineering is to make target compound production the primary function of an organism. Presently this is not possible due a fundamental lack of understanding of evolved biological complexity. However, the use of evolution itself to achieve engineering objectives is a powerful asset that sets synthetic biology aside from other engineering disciplines [10].

The process of evolution can be used not only to produce superior strains but also to inform rational design. By sequencing the genes and genomes of cells isolated from evolving populations it is now possible to pinpoint the time at which advantageous mutations arise (Box 1). By interrogating the nature of evolved mutations using tools such as transcriptomics, proteomics, and metabolomics, genotype-phenotype relationships can now be understood with unprecedented clarity. This approach has been used to investigate the mechanisms of various stress tolerance phenotypes in industrial microorganisms, and is beginning to be implemented for the evolution of metabolically productive genomes.

Controlling Laboratory Evolution with Biosensors
Adaptive laboratory evolution (ALE) is a widely used and highly effective tool in metabolic engineering for creating industrial strains with superior properties (see Figure I in Box 1). If ALE could be used to evolve metabolic productivity, then many of the challenges of traditional metabolic engineering could be overcome. Productive strains could simply be evolved, reducing the significant time and cost currently necessary to achieve commercial yields. Most importantly, the evolutionary process could be documented using whole-genome sequencing and the tools of systems biology (Box 1). The interrogation of the genotype-phenotype relationship in evolved strains could then be used to inform rational design with completely novel engineering principles. For example, mutations in genes involved in cellular processes that are currently considered peripheral to metabolic productivity could be hugely important. It is possible that cellular processes/features such as ribosome biogenesis, the cell cycle, cellular morphology, or cell membrane composition greatly affect the metabolic flux towards a particular compound. The process of synthetic evolution would reveal such phenomena.

The most immediate challenge associated with using biosensors to evolve productive genomes lies in converting the concentration of a desired compound into an output for cell survival. Much of synthetic biology is concerned with the engineering of tailored responses to biological signals [11,12], and small-molecule biosensors have great potential for achieving these goals [13] (Box 2). The coupling of cell survival to target metabolite concentration is beginning to be realised in the form of in vivo biosensors (see Figure IA in Box 2), and these biosensors are now being used to select for novel and productive microbial genes and genomes.

Transcriptional Regulator-Mediated Biosensing
Most biosensors fall under two main categories, allosterically controlled transcriptional regulators (TRs), or RNA secondary structures with metabolite specific ligands (see Figure IB, C in Box 2). TRs are an obvious choice for metabolite biosensors because the bacterial domain of life is replete with small-molecule regulated transcriptional repressors and activators [14–16]. The

Glossary
Adaptive laboratory evolution (ALE): a process in which a dividing population of microorganisms evolves tolerance to a selection pressure over time.
Biosensor: any molecular device or structure that can sense a molecule of interest and output a detectable signal in response.
Fluorescence-activated cell sorting (FACS): individual cells can be separated and cultured from a mixed population based on their fluorescence.
High-throughout screening (HTS): where desired strain characteristics such as metabolic productivity can be selected from large libraries of genetically diverse cells.
Metabolic productivity: the capacity of a particular metabolic network to convert a carbon source into a metabolite of commercial interest.
Metabolite: an organic molecule involved in metabolism, many of which can be used in industry as biofuels, pharmaceuticals, or chemicals.
Ribosome binding site (RBS): a site in an mRNA molecule that enables translation via association with a ribosome.
Single-nucleotide polymorphism (SNP): caused by DNA mutagenesis or replication errors.
Titre: the concentration of a particular compound. In this article ‘titre’ refers to the concentration of an industrially important metabolite in a microbial culture.
Transcriptional regulator (TR): typically a protein that binds to DNA to promote the transcription of a downstream open reading frame (ORF).
Yellow fluorescent protein (YFP): used in the same way as GFP.
Yield: the amount of target metabolite produced per amount of carbon source provided for the microbial culture.
Box 1. Adaptive Laboratory Evolution (ALE)

There are many examples of the power of laboratory evolution to improve microbial cell performance. Both Saccharomyces cerevisiae (yeast) and Escherichia coli have been evolved to have increased heat tolerance [36], altered substrate specificity [37–39], increased tolerance to toxic compounds [40–43], as well as numerous other phenotypes [44]. These outcomes are typically achieved by exposing continuously dividing populations of cells to increasingly stressful conditions over time. During this process individual cells in a population randomly accumulate genetic mutations either as a consequence of natural DNA replication errors or via a mutagenesis mechanism imposed by the researcher. By chance, some mutations encode phenotypic changes that allow cells to better tolerate the stress condition, enabling them to grow and divide faster than other cells in the population (Figure I). Eventually, subpopulations with advantageous mutations can take over a population such that the original ‘parent strain’ is no longer present. As the stress is increased, further beneficial mutations can be selected for until either the desired objective is met or no further stress can be tolerated. This type of adaptive laboratory evolution has been highly successful and widely adopted because the desired strain characteristic is naturally coupled to cell survival.

Figure I. ALE. (A) A host microorganism is continuously grown under an industrially relevant selection pressure such as altered temperature, carbon source, or in the presence of a toxic compound. Individual cells in the population randomly accumulate mutations (blue- and red-coloured cells) through DNA replication errors, and by chance some of these mutations enable the cells to grow and divide more rapidly than others in the population. (B) Throughout the evolution experiment individual colonies can be isolated on solid media such that sequencing of their genomes (C) can be used to determine the relationship between particular mutations and the adaptive phenotype. Candidate mutations can be identified by aligning reads to a reference genome and determining the relationship between evolved genomes and strain fitness (C). Potentially beneficial mutations are ‘reverse engineered’ into the parent strain to empirically determine their contribution to the phenotype of interest (D).

classic example of such a regulatory protein is the Lac repressor, which represses the expression of the lac operon by binding to operator sequences near the lac promoter. Binding of allolactose by the Lac repressor causes a conformational change that releases the repressor protein from the DNA, allowing transcription of the lac operon to proceed [17].

RNA-Mediated Biosensing

RNA biosensors are also widely distributed in nature, especially in the bacterial domain as riboswitches [18,19]. Riboswitches modulate mRNA secondary structure in the 5’ untranslated region (UTR) such that the accessibility of a ribosome-binding site (RBS) to the ribosome is
Box 2. Small-Molecule Biosensors

There has been a recent surge in the development of small-molecule biosensors that form part of HTS platforms [45]. In these scenarios genetic diversity is introduced into a population of potential producers via classical mutagenesis methods such as ultraviolet irradiation or ethyl methanesulfonate (EMS) treatment [46]. Every cell in the population expresses a small-molecule biosensor that can detect the target production compound and enable the expression of a GFP or antibiotic resistance gene in proportion to target compound concentration (Figure 1A). Individual cells or subpopulations that have the highest target-molecule productivity can then be selected for the ability to grow in the presence of antibiotic, or isolated using FACS. Small-molecule biosensors most commonly take the form of allosterically regulated TFs (Figure 1B) and RNA secondary structures such as riboswitches (Figure 1C). The survival/selection mechanisms are completely modular and are compatible with any type of small-molecule detection device, and GFP fluorescence and antibiotic resistance merely serve as illustrative examples.

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<th>Signal processing</th>
<th>Output</th>
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<td>(A) Metabolite concentration</td>
<td>Biosensor</td>
<td>Selection and survival</td>
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<td>(B) Transcriptionally regulated biosensor</td>
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<tr>
<td>Metabolite</td>
<td>TR</td>
<td>Biosensor</td>
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<tr>
<td>Promoter</td>
<td>GFP</td>
<td>Selection and survival</td>
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<tr>
<td>(C) RNA riboswitch biosensor</td>
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<tr>
<td>Metabolite</td>
<td>RBS</td>
<td>Antibiotic resistance gene</td>
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<tr>
<td>Promoter</td>
<td>ORF</td>
<td>mRNA</td>
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Figure 1. Biosensor-Mediated Selection for ALE. (A) A generalised situation where the concentration of a desired metabolite is detected using an in vivo biosensor. In the presence of target compound the biosensor actuates an output that can be used to select productive cells from a mixed population. (B) An allosterically controlled TR protein binds to its target promoter sequence only in the presence of target compound, initiating the expression of a GFP. Highly fluorescent cells (coloured green) can then be selected from the population using FACS. (C) An example of a riboswitch biosensor where an RNA secondary structure exists in the 5′-UTR of the mRNA for an antibiotic resistance gene. In the absence of target metabolite the RBS is located in the RNA secondary structure making it inaccessible to the ribosome, therefore preventing translation. Through specific binding of the target metabolite to the riboswitch RNA, a conformational shift occurs that exposes the RBS, allowing translation and therefore antibiotic resistance gene expression. Only cells that produce enough target metabolite to cause the riboswitch to change conformation can survive in a culture that is exposed to antibiotic, enabling the selection of high producers (green) from a mixed population. The GFP and antibiotic resistance expression cassettes are interchangeable between different types of biosensor, and are shown separately here for clarity.

dependent on the presence of a small molecule (see Figure 1C in Box 2). Binding of the target small molecule changes the conformation of the riboswitch, altering RBS availability and therefore the initiation of translation. Other RNA biosensors use catalytic RNA structures called ribozymes that are capable of splicing their own mRNA transcript. When a small-molecule binding RNA domain such as an aptamer [20] is connected to a ribozyme, the presence of small molecule can cause a conformational shift that prevents the self-cleaving activity of the ribozyme.
Aptamer domains can be evolved for potentially any metabolite using the systematic evolution of ligands by exponential enrichment (SELEX) technique [20]. Unfortunately, the fact that aptamers are created in vitro means that they seldom have the same properties and folding conformations when expressed in vivo due to differences in pH and salt concentrations that are found in the cytosol [21].

**Biosensor-Mediated High-Throughput Screening**

A selection of the most successful transcription factor and RNA biosensors and their effectiveness in implementing small-molecule productivity are highlighted in Table 1. It is noteworthy that most of these examples use biosensors for high-throughput screening (HTS) of mutant or producer-strain libraries. These strategies are extremely effective at screening for specific biological parts such as enzymes or RBSs [22] from mutated libraries. Enzymes that survive the selection process often have novel mutations and dramatically improved kinetics [23–26] and even altered substrate specificity [27]. HTS has also been executed at the whole-genome level [28], with random mutations being introduced and biosensor-mediated selection being employed to isolate superior producers for a variety of products [28]. An excellent example of this process is the selection of superior L-lysine-producing strains of *Corynebacterium glutamicum* [29] where a naturally occurring L-lysine-regulated transcription factor (LysG) was used to regulate the expression of a yellow fluorescent protein (YFP) from the LysG-responsive lysE promoter. Fluorescence-activated cell sorting (FACS) screening was then successfully used to select high producers from a population that had been chemically mutagenised.

**Biosensor-Mediated ALE**

For biosensor-mediated evolution to reach its full potential, whole genomes must be selected for and sequenced such that novel mutations and new ‘design principles’ for metabolic engineering and synthetic biology can be elucidated. Furthermore, the lower mutation rates that are obtained form natural DNA replication errors during ALE (as opposed to chemical or UV irradiation mutagenesis methods) are far more amenable to understanding genotype–phenotype relationships.

This phenomenon was demonstrated recently with the biosensor-mediated ALE of *C. glutamicum* for L-valine production [30]. The Lrp TR upregulated YFP expression from the brmFE promoter when bound by branched-chain amino acids. Five rounds of FACS with the top 10% of YFP producers being selected resulted in a 25% increase in L-valine production and a fourfold decrease in byproduct formation. Genome sequencing revealed that a loss-of-function single-nucleotide polymorphism (SNP) in a urease accessory protein gene (*UreD*) and a mutation in a global regulator protein (*GlxR*) were responsible for the production phenotype. Another recent example of biosensor-mediated evolution was carried out in *E. coli* for the production of isopentenyl pyrophosphate (IPP) [31]. A system termed ‘feedback-regulated evolution of phenotype’ (FREP) was developed where the genome-wide mutation rate is proportional to the intracellular IPP concentration via the expression of a dysfunctional DNA proofreading enzyme. Only once production levels have increased does the mutation rate and subsequent lethality decrease, allowing productive strains to stabilise in a phenotypically diverse population. The production of lycopene, a metabolite downstream from IPP in the isoprenoid pathway, was used to assess pathway productivity over the course of a 432 h evolution experiment. Whole-genome sequencing of an evolved strain indicated that several mutations in genes encoding ribosomal RNA as well as translation initiation and RNA catabolism were associated with productivity. Amazingly, even though lycopene production increased 16-fold due to the biosensor-mediated evolution process, only one gene known to increase yield was mutated (aceE). These results illustrate the potential that adaptive evolution has for revealing non-obvious engineering targets.
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<th>Target Phenotype</th>
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</table>

*Abbreviation: N/a, not applicable.
One of the principal barriers to the success of biosensor-mediated ALE is the proliferation of evolutionary ‘cheaters’ in a population [32]. In theory it is possible for mutations to occur in biosensor components, such as green fluorescent protein (GFP) or antibiotic resistance genes or the promoters that regulate their expression, such that the biosensor output is increased even though the biosensor input (metabolite concentration) has not. In the case of lycopene production in E. coli [31], periodically plating for individual colonies during the evolution experiment, and using only the highest lycopene producers (determined by red colony colour) to continue the experiment alleviated this issue. The problem of ‘cheater’ evolution was also grappled with in a recent biosensor-mediated evolution approach aimed at improving naringenin and glucaric acid production in E. coli [32]. Instead of relying on random mutations, multiplexed automated genome engineering (MAGE) [33] was used to target specific metabolic genes for mutation. In addition, alternating between positive and negative selection pressure was used to eliminate cheaters from a population during each round of mutagenesis and screening. This approach was effective at screening libraries of up to 1 billion cells and resulted in record titres of naringenin and a 36-fold improvement in glucaric acid production. An ideal biosensor can detect the target metabolite through a range of concentrations that are relevant to both strain development and commercial production titres. This would require a sensor to respond to metabolite concentrations over three orders of magnitude typically ranging from low micromolar to high millimolar levels. This study also demonstrated great insight into the effective functional ranges of detection for various biosensors in E. coli, and explored methods for fine-tuning the dynamic range of expression for TR/promoter pairs [32].

Box 3. Cell Encapsulation and Extracellular Biosensing

There are many conceivable strategies that can be used to avoid the evolution of cheaters within a biosensor-regulated evolution set-up. The ultimate solution to the cheater problem, however, may be to impose an extracellular selection pressure that is not subject to evolution. In theory this can be achieved by using a fluorescent reporter outside the cell, which when encapsulated in an oil droplet can be sorted according the amount of target compound being excreted (Figure I). FADS [70] has recently been used to screen a mutant library of 10^6 yeast cells for improved α-amylase enzyme expression and secretion. Cells were encapsulated in droplets along with an enzyme substrate that fluoresces when cleaved by α-amylase, enabling superior mutants to be selected from the population [71]. FADS has also been developed as a platform for screening individual cells based on the concentration of extracellular xylose and lactate [72]. Metabolite detection was achieved by using enzyme assays that have fluorescence outputs and are contained within the extracellular droplet environment. FADS technology holds great promise for the evolution of productive microbial genomes, but in common with other approaches is currently limited by the available detection mechanisms for small molecules.

Figure I. Cell Encapsulation and FADS. By encapsulating single cells in emulsion droplets the concentration of extracellular proteins or metabolites can be selected for using FADS.
One promising solution to the problem of cheater evolution is to employ a biosensor that is not subject to evolution. This can be achieved by using a non-genetically encoded biosensor outside the cell such that secreted product titre can be detected (Box 3). Product concentrations are connected to individual cells in a population through encapsulation in oil/water emulsion droplets, which can be analysed using fluorescence-activated droplet sorting (FADS) technology (see Figure 1A in Box 3). Because the biosensor is not genetically encoded by the cells undergoing evolution, it is impossible for cheaters to arise in the population. The challenge for the progression of this technology lies in the creation of extracellular biosensors that can detect any metabolite of interest. Synthetic protein switches [34,35] are one class of biosensors that are particularly well suited to this application because of their in vitro functionality and high degree of modularity.

Concluding Remarks
The sustainable production of transportation fuels, industrial chemicals, and pharmaceuticals is essential for the establishment of an environmentally sustainable economy. The tools of synthetic biology and metabolic engineering are set to play a leading role in this transition because they can be used to create microbial strains that convert renewable carbon sources into fuels, chemicals, and pharmaceuticals. Redesigning microbial metabolism to achieve meaningful production levels is fraught with challenges that are imposed by the inherent complexity of biological systems. However, the stunning diversity and functionality that exists in the biosphere hints at what can be achieved with synthetic biology if the power of evolution can be harnessed (see Outstanding Questions). This ‘synthetic evolution’ approach is beginning to emerge at the intersection between the fields of synthetic biology and metabolic engineering where small-molecule biosensors are being used to evolve metabolic productivity. Ideally, the evolution of industrially-relevant metabolite productivity will enable the replacement of fossil fuels and petrochemicals while at the same time informing rational design in synthetic biology and improving biological knowledge.

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