Graphical abstract

Systems-scale

Synthetic biology-accelerated directed evolution cycles

Complex multi-genic phenotypes

Title: Directed evolution and synthetic biology applications to microbial systems

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Abstract

Biotechnology applications require engineering complex multi-genic traits. The lack of knowledge on the genetic basis of complex phenotypes restricts our ability to rationally engineer them. However, complex phenotypes can be engineered at the systems level, utilizing directed evolution strategies that drive whole biological systems toward desired phenotypes without requiring prior knowledge of the genetic basis of the targeted trait. Recent developments in the synthetic biology field accelerates the directed evolution cycle, facilitating engineering of increasingly complex traits in biological systems. In this review, we summarize some of the most recent advances in directed evolution and synthetic biology that allows engineering of complex traits in microbial systems. Then, we discuss applications that can be achieved through engineering at the systems level.

Introduction

Microbes have been successfully employed in a broad range of applications, including in the food, pharmaceutical, petrochemical and bioremediation industries [1]. Increasingly complex applications are demanding more sophisticated designs, that consider optimization of multiple phenotypes such as tolerance, pathway flux, and growth rate, each of which is governed by multiple genes and their interaction with environmental factors. The lack of knowledge on the genetic basis of these complex traits restricts our ability to rationally engineer them. An alternative approach to manipulate these complex phenotypes is to engineer at the systems level [2], driving entire systems toward desired phenotypes without deep *a priori* knowledge of the underlying mechanisms.

Recent technological developments facilitates engineering of microbes at the systems level. These advances come primarily through the fields of directed evolution, metabolic engineering, and synthetic biology, with successful applications already at the gene, pathway, genome and even multi-organism (consortia) level. This approach sets the stage to explore diverse and complex functions for novel biotechnology applications. In this review, we discuss strategies that can be used to engineer complex traits in microbial systems. Then, we provide examples and applications of biological systems engineering on increasingly complex scales.

Engineering complex traits in microbial systems

Engineering complex traits can be accomplished using directed evolution strategies [3]. Directed evolution mimics natural evolution in more controlled settings to evolve biological systems toward user-defined phenotypes. A common demonstration of this idea is present in adaptive laboratory evolution (ALE) experiments. During ALE, microbial cultures are grown from hundreds of generations in defined selective conditions, which allows evolution of improved phenotypes [4]. As an example, Tenaillon and collaborators utilized ALE to evolve temperature tolerance, a complex trait whose genetic basis are poorly understood. [5]. A number of studies applied ALE in engineered *E. coli* or yeast strains to successfully improve biofuels or chemicals yield and productivity [6–8].

To evolve increasingly complex phenotypes at reasonable timescales, directed evolution usually relies on cycles of diversity generation and artificial selective pressure to amplify improved variants. Hence, the success of directed evolution depends largely on two factors: the ability to generate functional **diversity and proper screening** or selection methods to identify variants with real phenotypic improvement.

Recently, a significant contribution to directed evolution strategies came from the burgeoning field of synthetic biology. This field aims to design, build, and improve biological components and systems that do not exist in nature [9]. In doing so, synthetic biology developed tools that can accelerate directed evolution by increasing the efficiency of diversity generation and by broadening the spectrum of screenable or selectable traits. In this section, we discuss strategies provided by directed evolution and synthetic biology that can be used to engineer complex traits in microbial systems.

Increasing genetic diversity

The realization that diversity is the fundamental basis of evolution dates back to Darwin. As such, multiple methods to increase genetic diversity is employed by directed evolution strategies. With increased diversity, more likely mutations that confer complex phenotypes could be present, most of which we would not be able to rationalize *a priori*. As an example, random diversity can be generated using epPCR, a method that relies on the fallibility of DNA replication, utilizing an error-prone, thermostable polymerase that lacks proofreading activity [10]. Using diversity generated by epPCR, Summeren-Wesenhagen and Marienhagen were able to optimize a rate-limiting enzyme from a pinosylvin pathway to increase titer to 91 mg/L [11].

Another frequently used method to generate diversity is site-directed mutagenesis (SDM), although this approach requires some structural information of the targets. SDM allows codon saturation of specific targeted sites [12], unraveling mutations that could improve the activity of interest. For instance, Bastian and collaborators used SDM to switch cofactor specificity of two key enzymes involved in isobutanol production, a leading candidate biofuel. By eliminating cofactor imbalance in the strain, the authors were able to maximize the theoretical yield of isobutanol production [13^{*}]. **Firnberg and Ostermeier developed a variation of SDM named PFunkel, which allows easy generation of custom libraries across multiple sites simultaneously [14]. The authors leveraged this strategy to construct a complete codon substitution library in a beta-lactamase gene and identified mutations conferring resistance to a clinically relevant inhibitor.**

Genetic diversity can be further increased using recent synthetic biology tools that allows a more efficient and focused generation of diversity towards desired targets to accelerate evolution (Figure 1). As an example, increased diversity at targeted sites can be easily accomplished in *E. coli* using Multiplex Automated Genome Engineering or MAGE [15^{*}]. Using recursive rounds of recombination-based engineering (recombineering), MAGE introduces chromosomal edits encoded in ssDNA oligonucleotides to generate combinatorial diversity across multiple levels on a systems-scale (Figure 1A). MAGE was successfully applied from pathway optimization [15–17^{*}] to recoding the *E. coli* genome [18,19^{**}]. A variation of MAGE was also developed for the yeast *Saccharomyces cerevisiae* (YOGES) [20].

Diversity can also be generated by combining mutations present in a population via recursive rounds of recombination at the genome-level, which is known as genome shuffling (Figure 1B) [21]. Genome shuffling greatly speeds the rate of evolution when compared to classic mutagenesis and screening, and has been extensively used to evolve traits in a variety of organisms and applications (recently reviewed [22]).

One of the most recent additions to the synthetic biology toolbox is the clustered regularly interspaced short palindromic repeats (CRISPR) system, whose endonuclease (most notably Cas9 from *Streptococcus pyogenes*) has been leveraged to introduce genome edits in several organisms [23,24]. CRISPR can increase diversity by altering expression levels in genome-wide loss-of-function [25] and gain-of-function [26] screens. Additionally, diversity can be introduced through precise genome edits via homologous recombination (Figure 1C) [27^{**}], as has been explored in recent saturation mutagenesis studies [28,29]. **In addition, CRISPR-**

Cas based engineering is easily multiplexable [23], which has been leveraged to introduce rationally designed edits ranging from point mutations to entire metabolic pathways [30].

Diversity can be increased even further by expanding the sequence space beyond the host genome. Recent synthetic biology tools contribute to that effort through utilization of non-standard amino acids to evolve new catalytic functions in proteins (recently reviewed [31]), fully orthogonal ribosome systems [32•] and by efficiently importing heterologous functions from targeted organisms [33] or metagenomic samples (Figure 1D) [34,35•].

Screening or selecting complex traits

These new synthetic biology tools **increase** our ability to generate mutations and diversify a population. **With increased diversity**, the limiting factor in directed evolution applications is how to **screen or select** for the improved variants in a population. If the trait of interest is linked to a fitness increase, then growth selections can be used. Alternatively, screening methods that rely on colorimetric assays or FACS based sorting can be employed (extensively reviewed elsewhere [36]). However, screening methods are available to only a few traits of interest. Synthetic biology can address this issue by developing new tools that broadens the spectrum of traits that can be selected or screened (Figure 2).

Synthetic biology can contribute to this step of directed evolution by developing biosensors (Figure 2A) that can detect the trait of interest and trigger expression of fluorescent proteins (screenable) or antibiotic resistance genes (selectable). Most biosensors are based on transcription factors and their cognate promoter pairs [17,37•], **although RNA-based devices have also been developed** [38]. Novel biosensors can be designed *de novo*, with successful examples for both RNA-based [39] and protein-based [40] sensors. Using biosensors, it is possible to optimize entire pathways and gene clusters. As a case in point, Raman and collaborators **used** MAGE to generate genome-wide targeted diversity **and then employed a biosensor** based selection strategy to enrich high producers. With this approach, they increased production of naringerin and glucaric acid by 36- and 22-fold, respectively [17•].

Biosensors can be integrated in genetic circuits (recently reviewed [41]) to expand the complexity of inputs that can be connected to desired outputs. Ultimately, more sophisticated genetic circuits could expand our ability to screen and **select** traits in two ways (Figure 2B). First, a single input can be connected to multiple outputs. In this case, a trait of interest could trigger a cascade of regulatory networks to rewire the cell toward a desired phenotype of interest, which ultimately is amenable to screening or selection. Rewiring the cell metabolism upon a specific input was recently leveraged to **produce isopropanol** when **a sufficient** cell density was achieved [42]. Second, multiple inputs can be connected to a single output through robust logic gates, which results in increased specificity for the phenotype being selected or screened. A recent demonstration of this concept used RNA thermosensors to build a 3-input genetic circuit that triggers GFP expression [43].

Another strategy for efficient selection is to link the trait of interest to a fitness increase. Phage-assisted continuous evolution (PACE) accomplished this by using a modified M13 phage cycle so that the trait of interest is linked to expression of a phage protein required for infectivity [44••]. Therefore, phage harboring improved variants will have increased fitness by infecting more cells in a “lagoon” of replenished *E. coli* cells, allowing continuous rounds of evolution

(Figure 2C). PACE was recently improved by including the ability to modulate the stringency of selection and to perform negative selection against undesired variants [45].

Applications of synthetic biology on a systems-level

With efficient diversity generation methods (Figure 1) and proper screening or selection strategies (Figure 2), rounds of directed evolution can be successfully employed to engineer complex traits in microbial systems even if the genetic basis are not fully understood. This brings exciting new frontiers on the range of functions and applications that biotechnology will be able to address in the near future. In this section, we describe applications of directed evolution and synthetic biology on increasing levels of microbial systems (Figure 3), providing examples and future challenges that must be addressed.

On a first level, proteins can be evolved for novel functions not previously encountered in nature. This can be accomplished through incorporation of non-standard amino acids. As an example, Xiao and collaborators employed non-standard amino acids to evolve a lactamase with improved catalytic efficiency that could not be reproduced by a mutation with standard amino acids [46]. Therefore, novel chemistries and properties can start to be explored by biological systems, and these novel functions can be implemented in pathways to achieve entirely new applications. Pathways with novel functions can then be optimized using described tools such as MAGE [15^{*}] or CRISPR-Cas [27^{**}], coupled with efficient screening or selection strategies. Additionally, recently developed methods like ePathBrick [47] and CasEMBLR [48] allow rapid pathway assembly and testing for desired functions. Therefore, complex new functions can be engineered at the pathway level using synthetic biology and directed evolution tools.

A challenge in engineering at the pathway level is dealing with the regulatory complexity inside the cell, which is often unknown. The engineering concept of modularity has been applied to synthetic biology to partially address this issue [1,49–51]. As a case in point, categorizing fatty acid biosynthesis in three distinct modules led to efficient optimization of production in *E. coli* [52]. By optimizing transcription and translation levels of each module, the authors were able to balance intermediates production and consumption to eliminate bottlenecks.

This modular engineering strategy has also been applied on bigger scales. To overcome native regulatory mechanisms, Temme and collaborators refactored a 23.5 kb gene cluster responsible for nitrogen fixation to ammonia in *Klebsiella oxytoca* [53]. **The refactored gene cluster was controlled by defined synthetic elements such as well-characterized promoters and RBSs. After refactoring, the authors were able to transfer the functional cluster to *E. coli*.** A more recent example refactored the photosystem II from the alga *Chlamydomonas reinhardtii*, demonstrating exchange between algae species [54]. A promising application of refactoring strategies is the combinatorial biosynthesis of small molecules [55]. Libraries of such molecules could constitute a new generation of therapeutics.

Genome-scale engineering is a promising application of synthetic and systems biology. With tools to edit targeted sites on a multiplex fashion, recoded organisms with altered genetic codes have been demonstrated [18]. A recent application relied on such altered genetic codes to engineer a metabolic dependence on non-standard amino acids for survival [56], with implications for GMO containment. Additionally, availability of efficient DNA assembly methods

coupled with decreasing DNA synthesis cost is leading to synthesis of entire genomes *de novo* [57]. This can be explored to synthesize refactored genomes with reduced complexity, which would facilitate studying and further manipulating these systems. The complete synthesis of a functional yeast chromosome with designed changes exemplifies this concept [58^{••}], and computer-aided tools to design refactored genomes are already available [59]. These advances provides a foundation for the ability to rationally design and order customized genomes in the future, which would revolutionize biotechnology and the biological sciences in general. However, understanding the rules regarding genome design and booting genomes into cells is yet to be elucidated.

Finally, synthetic biology can also be applied at the multi-organism level to engineer consortia. Engineering microbial consortia can expand metabolic capabilities through division of labor, reducing many limiting factors of pure cultures such as metabolic burden and toxic intermediate accumulation. This requires efficient cell-to-cell communication, which can be achieved using metabolic cross-feeding [60[•]] or genetic circuits based on quorum sensing signaling molecules [61]. A hallmark study engineered a fungal-bacterial consortia to produce isobutanol directly from cellulosic biomass [62^{••}]. Also, *E. coli* co-cultures have recently been applied to produce complex chemicals from a glucose/xylose sugar mixture [63]. Ultimately, the ability to predict and engineer complex interactions among organisms can substantiate several biotechnology applications from chemical production and bioremediation to human health. A promising field involves engineering the gut microbiome, which can potentially treat a broad range of human diseases. However, developing synthetic biology tools for non-model organisms and a deeper understanding of gut microbial interactions are required before this can be accomplished.

Conclusions and future perspectives

The ability to engineer complex multi-genic phenotypes is fundamental for several biotechnology endeavors and currently restricts the field. The combination of recent synthetic biology tools with directed evolution and metabolic engineering can allow engineering of biological systems as a whole, via manipulation of increasingly complex traits. With these technologies, complex systems and traits are now amenable to engineering, fueling novel biotechnology applications across multiple industrial sectors. **Rational diversity can now quickly and efficiently be generated using these recent synthetic biology tools, accelerating the directed evolution cycle.** Additionally, development of more characterized biosensors and robust genetic circuits will enhance screening and selection of improved variants. These advances will further accelerate directed evolution and increase the complexity of biotechnology designs in the near future.

Acknowledgements

We would like to thank the Gill lab for discussions on the topic. We also thank the US Department of Energy (Grant DE-SC0008812) and CAPES foundation for funding this work.

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Figures legends

Figure 1. Accelerating directed evolution through increased diversity generation. Four distinct synthetic biology tools that can increase genetic diversity are highlighted. A) Multiplex Automated Genome Engineering (MAGE) can generate combinatorial mutations in targeted genomic sites. B) Genome shuffling can combine mutations from different populations using recombination at the genome-level. C) CRISPR-Cas technologies allow designed edits to be incorporated into the genome via homologous recombination, a process that can easily be multiplexed. D) Heterologous functions can be imported and efficiently expressed into the host strain using heterologous sigma factors.

Figure 2. Accelerating directed evolution through efficient screening and selection strategies. Three distinct synthetic biology tools that can broaden the spectrum of screenable or selectable traits are highlighted. A) Novel biosensors can allow detection of metabolites of interest and trigger expression of screenable or selectable markers. B) Genetic circuits can increase the complexity of inputs (targeted phenotype) that can be connected to a desired output (screenable or selectable phenotype). C) Phage-assisted continuous evolution (PACE) links the trait of interest to a phage fitness increase to allow continuous rounds of evolution.

Figure 3. Applications of directed evolution and synthetic biology on a systems level. Multiple scales of biological systems can be engineered using directed evolution and synthetic biology tools. Increasing levels of biological systems (left) and engineering strategies (right) are highlighted.

Figure 1

Figure 2

Figure 3

genes and proteins
pathways
gene clusters
whole genomes
multi-organism

non-standard amino acid incorporation

Pathway assembly and optimization

Refactored gene clusters

Refactored genomes

Engineering consortia