Unlocking Nature's Biosynthetic Potential by Directed Genome Evolution

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Highlights

- Directed genome evolution is a powerful tool to unlock microbial biosynthetic potential.
- Adaptive laboratory evolution is a traditional but efficient tool to improve cell fitness.
- Various genome-scale engineering tools have been developed for targeted direct evolution.
- Emerging new technologies such as machine learning and automation can accelerate directed genome evolution.

Abstract

Microorganisms have been increasingly explored as microbial cell factories for production of fuels, chemicals, drugs, and materials. Among the various metabolic engineering strategies, directed genome evolution has emerged as one of the most powerful tools to unlock the full biosynthetic potential of microorganisms. Here we summarize the directed genome evolution strategies that have been developed in recent years, including adaptive laboratory evolution and various targeted genome-scale engineering strategies, and discuss their applications in basic and applied biological research.

Introduction

The current sustainability crisis caused by the depletion of petroleum resources and intensifying environmental problems (e.g. global warming) have heightened interest in converting renewable raw materials into fuels, chemicals, drugs, and materials using microbial cell factories [1,2]. Most of the existing metabolic engineering strategies used to develop microbial cell factories involve the use of various omics tools and/or computational modeling tools to identify genetic targets responsible for a novel or improved phenotype followed by genetic manipulations such as overexpression, downregulation, and knockout of these target genes [3,4]. However, such rational design strategies are time-consuming and not always effective because identifying gene targets for genetic manipulation remains an overwhelming challenge due to the complexity and interconnectivity of metabolic networks [5,6].

To address these limitations, an alternative metabolic engineering strategy is to mimic the Darwinian evolution process in the test tube consisting of iterative rounds of mutagenesis and screening or selection on the genome scale. This directed genome evolution strategy has emerged as a powerful tool in strain development because it can facilitate high-throughput genotyping and phenotyping, which significantly accelerates our understanding and engineering of microbial genomes [5]. Directed genome evolution can be performed in two manners: random and targeted. The random approach usually refers to adaptive laboratory evolution (ALE)-based random mutagenesis, which is achieved through prolonged culturing of cells under specified conditions to naturally select for the beneficial mutations [7,8]. Unlike the random approach, targeted directed evolution results in elaborate and massive traceable genome modifications, and can be broadly classified into three categories: genome editing, transcriptome engineering, and genome synthesis [5].

In this review, we highlight the recent development and application of directed genome evolution strategies and tools for metabolic engineering (Table 1). We also discuss their pros and cons and the new biological insights they have uncovered. Finally, we offer our future perspectives on directed genome evolution.

Directed genome evolution in a random manner

Adaptive laboratory evolution (ALE) allows the metabolic engineering of microorganisms by combining genetic variation with selection of beneficial mutations in an unbiased fashion [9], and has proven highly effective in improving microbial phenotypes and investigating evolutionary phenomena on a genome-scale [10•]. As shown in Figure 1, ALE experiments are commonly performed in a controlled laboratory setting, under a clearly defined condition for prolonged periods of time, in the range of weeks to years to achieve the desired phenotypes. Thanks to their simple nutrient requirements, easy laboratory cultivation, and fast growth rate, microbial cells offer important advantages for ALE studies [7], and their biosynthetic potentials are increasingly discovered with the help of ALE and other high-throughput technologies such as next-generation sequencing (NGS) [11,12] and genotype-phenotype mapping [13]. Moreover, the database ALEdb (http://aledb.org) has collected over 130,295 mutations from the literature over the years (as of June 15, 2020), providing an informative platform for accessible consolidated ALE mutations [14]. Since several recent reviews have comprehensively summarized and discussed the strategies and applications of ALE [10•,15,16], we herein highlight only a few latest examples of how ALE unlocks the biosynthetic potential of microorganisms by improving cellular fitness or chemical production.

Enhancing cell growth. Cell growth rate is generally the most important fitness determinant in ALE experiments [10•]. Cho and coworkers deployed ALE to re-optimize growth performance of a genome-reduced (deletion of 1.1 Mbp) *Escherichia coli* strain. After 807 generations of ALE, the evolved strain exhibited a comparable growth rate to wild type in minimal medium [17]. Milo and coworkers recently constructed an autotrophic *E. coli* strain that could generate biomass carbon from CO_2 (by co-expressing Rubisco and phosphoribulokinase) and utilizes formate (oxidized by heterologous formate dehydrogenese) to provide reducing power and energy; the autotrophic growth was achieved following ~200 days of chemostat-based ALE (Table 1) [18••]. Meanwhile, the methylotrophic yeast *Pichia pastoris* was also converted into an autotroph by engineering the peroxisomal methanol-assimilation and CO_2-fixation pathways; the growth rate was improved more than 2-fold through ALE with CO_2 as a sole carbon [19•]. The unlocked CO_2-utilizing potential may provide the basis of a platform for producing chemicals and enzymes from CO_2.

Increasing tolerance. ALE has been established as an efficient tool to overcome stress and harsh conditions, such as growth inhibitors (byproducts from biomass pretreatment), high salinity, low pH, and high temperature. Through ALE, in combination with physiological characterization and omics analysis, Trinh and coworkers elucidated how *Yarrowia lipolytica* restructures its membrane to tolerate different types of ionic liquids (ILs) at high levels up to 18% (v/v), and discovered that sterols play a key role for exceptional IL tolerance [20]. Ren and coworkers performed ALE under high salinity stress to alleviate oxidative damage and improve lipid biosynthesis, and the final *Schizochytrium* sp. strain (obtained after 150 days) showed a 32.7% and 53.31% increase of cell dry weight and lipid yield, respectively [21]. Chen and coworkers also found that ALE conferred tolerance to dicarboxylic acids in *Saccharomyces*

cerevisiae by upregulating specific multidrug resistance transporters (e.g. Qdr3p) whose overexpression also improved muconic acid production (Table 1) [22•]. Because thermotolerant microorganisms are preferred for some industrial applications, Li and coworkers conducted ALE of a *S. cerevisiae* laboratory strain with stepwise temperature increases. The resulting strain could grow at 42 ℃, and the causative high-temperature growth (Htg^+) mutations, including exonic mutations in the SWI/SNF complex and F-type ATPase, were identified [23].

Improving chemical production. ALE has also been employed to improve the production of metabolites. Xing and coworkers developed a maltose utilization-based selection circuit: in this circuit, the L-tryptophan (L-Trp) biosensor activates the transcription of *malQ* (encoding a maltose hydrolase) by positively responding to the intracellular concentration of L-Trp. With generations of evolution, the resulting strains showed >65% increased L-Trp production [24]. A GREACE (genome replication engineering assisted continuous evolution)-assisted ALE was introduced to the endpoint fermentation broth of *E. coli* and enhanced lysine production by 14.8% to 155.0 g/L in 5-L batch fermentation (Table 1) [25]. Recently, Yang and coworkers constructed a coenzyme A (CoA)-dependent acetone-butanol-ethanol (ABE) pathway in *Clostridium cellulovorans*, which could produce n-butanol using agricultural waste as the sole carbon source. The ALE approach was adapted to strengthen the n-butanol tolerance of *C. cellulovorans* to maximize its n-butanol output potential, with the final engineered strain exhibiting a 138-fold improvement upon n-butanol production [26].

In addition, ALE experiments were employed to obtain and improve other microbial phenotypes, such as resolving energy depletion to maintain high aromatic metabolite phenotypes [27], overcoming flux limitation via desensitizing allosteric inhibition [28], and adapting cells to utilize non-native substrates [29]. Collectively, as a random directed genome evolution tool,

ALE can generate microbial cell factories with desired features through iterative rounds of acquiring beneficial mutations under specific conditions. Moreover, ALE can integrate with rational metabolic engineering strategies or targeted directed genome evolution to gain novel characteristics and thus perform more complex applications.

Directed genome evolution in a targeted manner

Directed genome evolution can be implemented on a genome-scale in a targeted manner in which each gene may be genetically modified individually or combinatorially. Compared to ALE, targeted directed genome evolution offers several advantages. Targeted directed genome evolution can efficiently and precisely construct mutant libraries covering entire genomes. Moreover, the majority of approaches for targeted directed genome evolution can be performed on a short timescale, while ALE requires days or even months of adaptation in the appropriate medium. Finally, whole-genome sequencing is necessary to detect mutations that appear over the course of ALE, while targeted directed genome evolution tools typically include traceable barcodes in the plasmids, allowing one to track all the genetic mutations and modifications on a population scale using high-throughput sequencing [30].

Targeted directed genome evolution methods enable feasible and efficient genome-scale engineering of microorganisms and help unlock their biosynthetic potentials. For example, **TRMR** (trackable multiplex recombineering) can knock down or overexpress virtually all genes in the *E. coli* genome and allows gene-trait mapping of those genetic modifications [31]. **TRACE** (tracing combinatorial engineered libraries) allows mapping of up to millions of combinatorially engineered genomes simultaneously and was used to explore growth selection dynamics in *E. coli* [32]. In addition, some tools for chromosomal integration of large

biosynthetic gene clusters (BGCs) were developed to assist natural product discovery and engineering. For example, **aMSGE** (advanced multiplex site-specific genome engineering) [33] and **CRAGE** (chassis-independent recombinase-assisted genome engineering) allow for efficient and accurate integration of BGCs [34•]. Furthermore, **YOGE** (yeast oligo-mediated genome engineering) is a method for oligonucleotide-mediated recombineering in *S. cerevisiae* and involves knocking out of *MLH1* and *MSH2* to increase oligonucleotide incorporation, overexpression of DNA recombinase, and optimizing transformation and oligonucleotide designs [35]. **GROs** (genomically recoded organisms) were created by replacing all instances of UAG stop codons with the synonymous UAA codons [36]. The UAG stop codon could then function as a sense codon for incorporation of non-standard amino acids. This approach was used to construct synthetic auxotrophs whose growth depended on the supply of synthetic amino acids [37].

Here, we will focus on the four different categories of targeted directed genome evolution strategies: oligo-based, RNA-based, clustered regularly interspaced short palindromic repeats (CRISPR)-based, and recombinase-based approaches (Table 1 and Table 2).

Oligo-based genome-scale engineering. **MAGE** (multiplexed automated genome engineering) enables multiplex mutagenesis of several target sites and genetic modifications with high efficiency in bacteria [38]. MAGE employs recombineering to incorporate mutagenic single-stranded DNA oligonucleotides (ssODNs) into the lagging strand of the replication fork during DNA replication, generating combinatorial genomic libraries. Since short oligonucleotides are cheap and DNA double-strand breaks are not required, MAGE is a rapid and efficient tool for genome-scale engineering. However, it cannot be easily adapted to higher eukaryotic organisms because of their slow growth rates, low allelic replacement efficiency, and

more complex DNA replication mechanism. Several MAGE-based strategies were subsequently developed, including **CoS-MAGE** (coselection MAGE) [39], **Microarray-Oligonucleotide (MO)-MAGE** [40], **eMAGE** (eukaryotic multiplexed automated genome engineering) (Table 1) [41], and **pORTMAGE** (portable MAGE) [42].

***RNA-based genome-scale engineering*.** Trans-acting regulatory RNAs can be employed for genome-scale screening of genes that improve production of the desired products [43]. A genome-scale synthetic sRNA (small RNA) library targeting 45% of genes in *E. coli* and a malonyl-CoA biosensor were constructed and used to screen for gene knockdown targets that improved malonyl-CoA production [44•]. **RAGE** (RNAi-assisted genome evolution) is a genome-wide engineering method that can continuously enhance desired traits through accumulation of beneficial downregulations of genes [43]. RAGE was used to identify yeast mutants with improved xylose utilization (Table 1) and a new determinant of furfural tolerance [45,46]. Furthermore, RNAi was combined with cDNA overexpression to enable genome-wide gain-of-function and loss-of-function modifications, and the genome-scale engineering process was further automated using an integrated robotic system [47]. RNA-based genome-wide engineering can generate pooled library of repression with high coverage and does not cause permanent change in the genome. Nevertheless, RNA-based gene knockdown depends on mRNA stability and usually has lower gene repression efficiency than the CRISPR interferences.

CRISPR-based genome-scale engineering. CRISPR based genome-scale engineering can easily generate high coverage of genome-wide libraries of deletion, activation, and interference. Consequently, it has become a preferred method for directed genome evolution even though two main limitations, i.e. possible off-targets and limited availability of high

throughput screening methods, still exist. A number of strategies have been developed (Figure 2), including:

(1) HR-mediated genome editing using CRISPR/Cas9. **CHAnGE** (CRISPR–Cas9- and homology-directed-repair-assisted genome-scale engineering) is a large-scale engineering tool that can readily produce a library of genome-wide gene knockouts in *S. cerevisiae*, disrupting more than 98% of target sequences with an average editing efficiency of 82% [48•]. **MAGIC** (multi-functional genome-wide CRISPR) system (Table 1) is another genome-scale engineering tool that combines the tri-functional CRISPR-AID system [49] with array-synthesized oligo pools [50••]. Pooled CRISPR interference (CRISPRi) screening of *S. cerevisiae* enabled by MAGIC is similar to genome-scale pooled-library CRISPRi screening developed for other microorganisms, such as *E. coli* [51] and *Vibrio natriegens* [52]. Both CHAnGE and MAGIC, in which the sgRNA on each plasmid functions as a unique barcode to track every mutant, have been used to identify new gene targets that conferred higher tolerance to furfural. More recently, **CasPER** (Cas9-mediated protein evolution reaction) is a method that employs error-prone PCR and Cas9-mediated genome integration to achieve 98-99% efficiencies for integration of mutant libraries of large-sized donor variants into single or multiple genomic sites. As proof of concept, *ERG12* and *ERG20*, two key genes in the mevalonate pathway, were selected for directed evolution using CasPER in *S. cerevisiae*, resulting in a double mutant strain with an 11-fold improvement in isoprenoid production [53].

(2) NHEJ-mediated genome editing using CRISPR/Cas9. *Y. lipolytica* is a non-conventional yeast for the production of lipid-based chemicals and biofuels. A genome-scale library of sgRNA targeting 7854 coding sequences in *Y. lipolytica* was created. The library was transformed into NHEJ-functional and NHEJ-disabled *Y. lipolytica* strains that constitutively

expressed Cas9. Because the most likely consequence of DNA double-strand breaks in a strain lacking NHEJ (the dominant DNA repair mechanism) is cell death, the cutting efficiency of each sgRNA could then be quantified. The authors were able to identify highly efficient sgRNAs that targeted 94% of genes in *Y. lipolytica* [54•].

(3) Base editing. Base editing is a CRISPR-based tool that can edit DNA without requiring DNA double-strand break or a donor template [55]. In this approach, Cas9 is fused with a cytidine deaminase enzyme, and the complex no longer induces DNA double-strand breaks but instead directly converts cytidine to uridine. **CRISPR-STOP** uses base editors to disrupt genes by mutating single nucleotide to create stop codons (Table 1). CRISPR-STOP is suitable for genome-scale engineering in human cells as the library of around 216000 unique inducible stop sgRNAs can potentially introduce stop codons in 17000 genes. Several technologies based on base editing were also developed, such as **iSTOP** (induction of stop codons) [56], TAM (targeted AID-mediated mutagenesis) [57], and CRISPR-X (using dCas9 to target activation-induced cytidine deaminase to enable point mutations) [58]. Base editing has been introduced to *E. coli* [59] and *S. cerevisiae* [60] for site-specific single nucleotide replacement and can be further adopted for genome-scale engineering.

(4) CRISPR/Cas9-based methods with short, trackable, integrated cellular barcodes. **MAGESTIC** (CRISPR–Cas9-based method for multiplexed accurate genome editing with short, trackable, integrated cellular barcodes) tags each guide-donor oligo with a unique genomic barcode (Table 1) [61•]. The plasmid barcode is integrated into the genome, while the residual guide-donor plasmid is eliminated via plasmid self-destruction, enabling high-throughput screening and phenotyping. **CREATE** (CRISPR-enabled trackable genome engineering) couples sgRNA with DNA donor template, which also functions as a unique barcode to map genotype-

phenotype relationships [62]. CREATE was used to improve lysine production in *E. coli* and to identify several mutations in lysine transporters, regulators, and biosynthetic genes, which contributed to the tolerance of S-2-aminoethyl-L-cysteine, a toxic analog of lysine [63]. **iCREATE** (iterative CREATE) was adapted from CREATE and included an additional gRNA plasmid removal step [64]. Iterative CREATE cycles were performed to obtain combinatorially engineered *E. coli* strains with improved glucose and xylose co-utilization and higher tolerance to furfural. In another study, the authors inserted an arabinose-inducible pBAD promoter upstream of the Cascade operon, *casABCDE* in an *E. coli* strain lacking Cas3 endonuclease [65]. The resultant strain was then used for CRISPR-based gene repression. A combinatorial library of sgRNA array plasmids was also constructed using Golden Gate assembly method. The system was used to screen for gene knockdown targets that helped increase production of malonyl-CoA and 3-hydroxypropionate.

(5) CRISPR/Cas9 and recombineering based technology. **CRMAGE** combines CRISPR/Cas9 system with λ Red recombineering-based MAGE technology and is an efficient method for genome engineering in *E. coli* (Table 1). DNA double-strand break induced by CRISPR/Cas9 system creates a selective pressure and stimulates the incorporation of single-stranded oligodeoxynucleotides (ssODNs). As a proof of concept, for *xylA* disruption, the recombineering efficiency using CRMAGE reached up to 99.7%, while only 0.6% efficiency was achieved by traditional MAGE. Furthermore, CRMAGE improved the efficiency of modulation of protein synthesis from 6% to 70% [66].

Recombinase-based genome-scale engineering. Synthetic chromosome rearrangement and modification by loxP-mediated evolution (**SCRaMbLE**) is a recombinase-based genome-scale engineering system [67]. LoxPsym, a palindromic DNA sequence, was inserted 3 bp

downstream of all non-essential genes. Cre recombinase then generated recombinations in either direction among LoxPsym sites. SCRaMbLE system has been introduced in the synthetic yeast genome (Sc 2.0), which enables generation of massive structural/genetic variations and potentially drives phenotypic evolution (e.g. increasing alkali tolerance) [68]. Several SCRaMbLE-based methods have been developed. For example, L-SCRaMbLE was developed in which a light-regulated Cre recombinase was utilized. Upon exposure to red light, the recombinase activity became tightly controlled with up to 179-fold induction [69•]. Reporter genes, ReSCuES, were included in the circuit to identify and select the strains that were SCRaMbLEd with high efficiency [70]. SCRaMbLE-in, a combinatorial method of *in vitro* recombinase toolkit and *in vivo* genome reshuffling system was developed for rapid pathway prototyping/optimization and chassis engineering (Table 1) [71]. Recombinase-based genome-scale engineering enables multiplex genome rearrangement that generates structural variation libraries. However, this method may not be applicable for slow growing organisms or organisms that do not use HR as DNA repair mechanism.

Conclusion and future perspectives

Directed genome evolution is a powerful tool to unlock microbial biosynthetic potential and improve desired phenotypes. With the development of novel strategies and tools for directed genome evolution, our ability to reprogram microbial cells for production of fuels, chemicals, drugs, and materials has been greatly expanded. Particularly, the combination of directed genome evolution with other emerging computational or high throughput technologies will not only significantly accelerate the metabolic engineering process but also provide new insights into cell metabolism and gene regulation. For example, genome-scale metabolic models that

computationally describe gene-protein-reaction associations can be used to predict the fitness effect of mutations, genetic interactions, evolutionary outcomes, and to decipher the mechanisms behind the mutational robustness [72,73]. Machine learning (ML) enables the identification of patterns within complex biological data across multiple scales and may accelerate the design of efficient and robust strains with optimized performance [74]. Microdroplet-assisted screening offers a high-throughput platform for secreted molecules with the speed of fluorescence-activated cell sorting and the phenotype-genotype linkage of microtiter plates, which can be used to improve biomolecule production and protein secretion [75•,76]. Furthermore, automation allows for complex culturing procedures or genetic manipulations that are too difficult or time-consuming to be performed manually, e.g. genome-scale engineering [47], and is being developed as an essential component of emerging biofoundries [77•,78].

The Design-Build-Test-Learn (DBTL) cycle is an increasingly adopted metabolic engineering framework that represents a more systematic and efficient approach to biosystem design or strain development [79]. Since no single technology can address all problems in the DBTL cycle, rational combination of the above-mentioned tools or strategies can fulfill this cycle and facilitate the development of a microbial cell factory with desired potential [80,81••]. Equally important to note is that other levels of directed evolution (e.g. protein and pathway) together with downstream processing/fermentation also play important roles on boosting the biosynthetic capabilities as well.

Conflict of interest statement

None declared.

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This study allows for attenuation of gene expression, using RNAi combined with high-throughput microfluidic single-cell screening for cells with improved protein secretion. By incorporating Cas9-mediated recombineering, the authors could tune the expression of the identified gene targets to further enhance protein production.

Figure captions

Figure 1. Scheme for adaptive laboratory evolution (ALE). (A) Microbes are cultured by serial dilution in shake flasks or chemostat bioreactor under the desired growth condition for a prolonged period of time to enrich the mutants with improved fitness. (B) Evolved strains are characterized for phenotypic improvements or increased fitness compared to the starting strain. (C) The single evolved clone is selected on predesigned plates. (D) Evolved strains are characterized by whole genome sequencing. (E) Mutations of evolved strains are revealed by comparative sequence analysis with starting strains; gene deletion and single nucleotide polymorphisms (SNPs) are the two common mutations. (F) Reverse engineering may be performed by introducing the mutations to starting strains to confirm the fitness increase.

Figure 2. Scheme for CRISPR-based targeted directed genome evolution. SgRNAs for genome-scale engineering are synthesized as oligos on microarray chips and cloned into the gRNA expression plasmids. The pooled plasmid libraries are transformed into the strain of interest constitutively expressing Cas9 for (A) gene deletion, (B) dCas9 for activation and (C) interference, or (D) nCas9 for base editing. The transformants are screened for the desired phenotypes. As genome sequencing is generally not required for targeted directed genome evolution, NGS is then used to identify the enrichment of guide sequences. The workflow can be iterated for several rounds to obtain complex phenotypes.

Table 1. Selected examples of directed genome evolution tools and applications.

Tools		**Applications**	**Reference**
ALE-based directed genome evolution	Growth-related	Achieved complete autotrophic growth (CO_2-utilizing) of *E. coli* strain after ~200 days of chemostat culture, and some mutations are linked to Calvin cycle	[18••]
	Tolerance-related	Conferred tolerance to dicarboxylic acids in *S. cerevisiae* by upregulating specific multidrug resistance transporters (e.g. QDR3)	[22•]
	Production-related	Improved lysine production by GREACE-assisted ALE in late fermentation broth, and mutations $SpeB^{A302V}$, $AtpB^{S165N}$ and $SecY^{M145V}$ mainly contributed to both improved cell integrity and lysine synthesis	[25]
Targeted directed genome evolution	Oligo-based	eMAGE: Rad51-independent gene editing enabled precise chromosome modifications at single base-pair resolution; used to diversifying β-carotene biosynthetic pathway in *S. cerevisiae*	[41]
	RNA-based	RAGE: Created a genome-scale library consisting of both genetic over-expression and down-regulation mutations in *S. cerevisiae* to improve xylose utilization rate	[45]
	CRISPR-based	(1) MAGIC: Combined CRISPR-AID and array-synthesized oligo pools to perform genome-scale gain-/reduction-/loss- of function in *S. cerevisiae*; used to identify genetic determinants of complex phenotypes, i.e. furfural tolerance (2) CRISPR-STOP: Used base editors to disrupt genes by changing single nucleotides to create stop codons; can easily introduce stop codon to ~17,000 human genes for functional screening (3) MAGESTIC: Used array-synthesized guide-donor oligos with genomic barcode for plasmid-based high-throughput editing and robust phenotyping in *S. cerevisiae* (4) CRMAGE: Combined CRISPR/Cas9 and λ Red recombineering based MAGE technology for efficient genome engineering of *E. coli*; improved the efficiency of modulation of protein synthesis to 70%	[50••] [55] [61•] [66]
	Recombinase-based	SCRaMbLE-in: included an *in vitro* recombinase toolkit for pathway prototyping and an *in vivo* genome reshuffling system for pathway integration; used to engineer pathways of β-carotene and violacein	[71]

Table 2. Pros and cons of various targeted directed genome evolution approaches.

Strategy	**Advantages**	**Disadvantages**
Oligo-based	• Short oligonucleotides are cheap and readily available • DNA double-strand break is not necessary	• Cannot be adapted to higher eukaryotic cells due to slow growth rates, low allelic replacement efficiency, and more complex DNA replication machinery
RNA-based	• Readily generate genome-scale library of repression with high coverage • No permanent change in the genome	• Lower gene repression efficiency than CRISPR interference • Dependent on mRNA stability
CRISPR-based	• Readily generate genome-wide libraries of deletion, activation, and interference with high coverage	• Possible off-target effects • Screening methods limited to growth or fluorescent-coupled phenotypes
Recombinase-based	• Large-scale genome rearrangement that creates a structural variation library • Multiplex replacement can be done simultaneously	• High death rate for haploid strains • Time consuming if the host is a slow growing organism • Only applicable to organisms employing HR as DNA repair mechanism

Figure 1

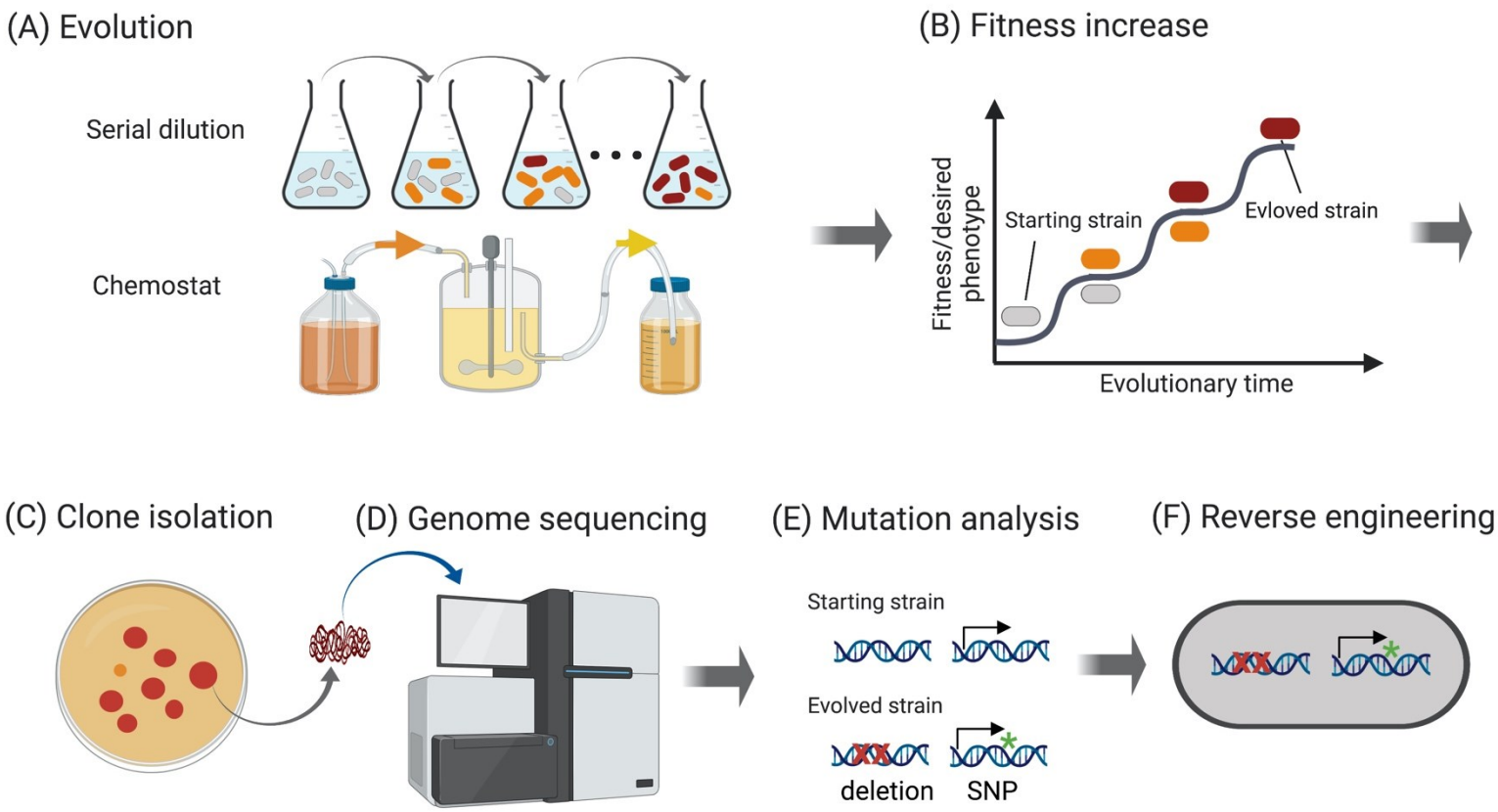
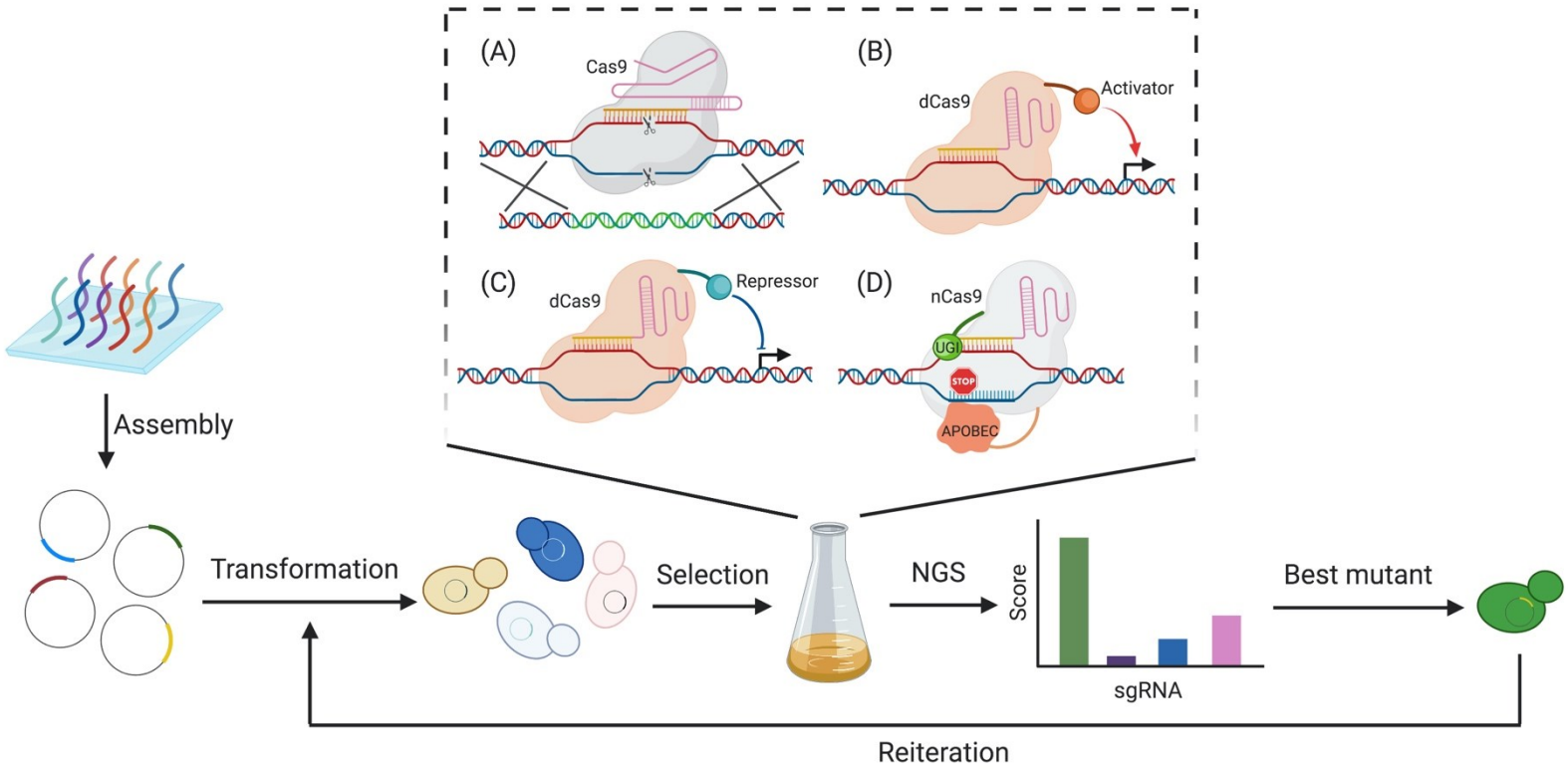

(A) Evolution
Serial dilution
Chemostat
(B) Fitness increase
Fitness/desired phenotype
Starting strain
Evloved strain
Evolutionary time
(C) Clone isolation
(D) Genome sequencing
(E) Mutation analysis
Starting strain
Evolved strain
deletion
SNP
(F) Reverse engineering

Figure 2


(A)
Cas9
(B)
dCas9
Activator
(C)
dCas9
Repressor
(D)
nCas9
UGI
STOP
APOBEC
Assembly
Transformation
Selection
NGS
Score
sgRNA
Best mutant
Reiteration