Journal of Industrial Microbiology & Biotechnology (2019) 46:1313–1326
https://doi.org/10.1007/s10295-019-02191-5

METABOLIC ENGINEERING AND SYNTHETIC BIOLOGY - REVIEW

Tools and systems for evolutionary engineering of biomolecules and microorganisms

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Received: 28 February 2019 / Accepted: 20 May 2019 / Published online: 27 May 2019

Abstract

Evolutionary approaches have been providing solutions to various bioengineering challenges in an efficient manner. In addition to traditional adaptive laboratory evolution and directed evolution, recent advances in synthetic biology and fluidic systems have opened a new era of evolutionary engineering. Synthetic genetic circuits have been created to control mutagenesis and enable screening of various phenotypes, particularly metabolite production. Fluidic systems can be used for high-throughput screening and multiplexed continuous cultivation of microorganisms. Moreover, continuous directed evolution has been achieved by combining all the steps of evolutionary engineering. Overall, modern tools and systems for evolutionary engineering can be used to establish the artificial equivalent to natural evolution for various research applications.

Keywords Evolutionary engineering · Mutagenesis · High-throughput screening · Selection · Continuous evolution

Introduction

Evolution enables living organisms to survive and adapt to changing environments. The mechanism underlying this process, descent with modification, not only shapes nature but also help researchers to engineer useful phenotypes. Adaptive laboratory evolution reproduces natural evolution in a research laboratory by passaging microbial cultures under selective pressures to which the cell must adapt [22, 48, 59, 66]. The evolutionary process has also been mimicked through directed evolution of enzymes [1, 14, 79], in which variants of a target gene are generated in vitro and transformed to cells and selected for the desired phenotype such as binding affinity. These evolutionary engineering approaches have emerged as important strategies for bioengineering. Numerous technologies are used in each step, with thenext-generation sequencing (NGS) technology enabling determination of genotype–phenotype relationships after the evolution [40, 71, 95].

In addition to the traditional technologies, recent advances in synthetic biology have improved the efficiency of evolutionary engineering and broadened the field to which these approaches can be applied. In vivo mutagenesis methods increased the library size and reduced the time and labor required to generate a library [105]. Genetically encoded biosensors have expanded the range of phenotypes that can be selected beyond the tolerance to stress and binding affinity of a ligand [51]. Microfluidic systems enabled high-throughput, single-cell screening [6, 43]. Synthetic genetic circuits and continuous evolution systems facilitated whole evolution cycles by reducing or even eliminating interventions by researchers.

In this review, we describe recent progress in evolutionary engineering of biomolecules and microorganisms (Fig. 1). The main objective of this article is to provide an updated view of the tools and systems useful for accelerating evolutionary engineering. Therefore, we focus on the methods used in each step of evolutionary engineering: diversity generation and phenotype screening. Thereafter, a more advanced concept for an efficient evolution known as continuous directed evolution will be explained that requires least interventions by researchers such as PCR, cloning, and transformation.

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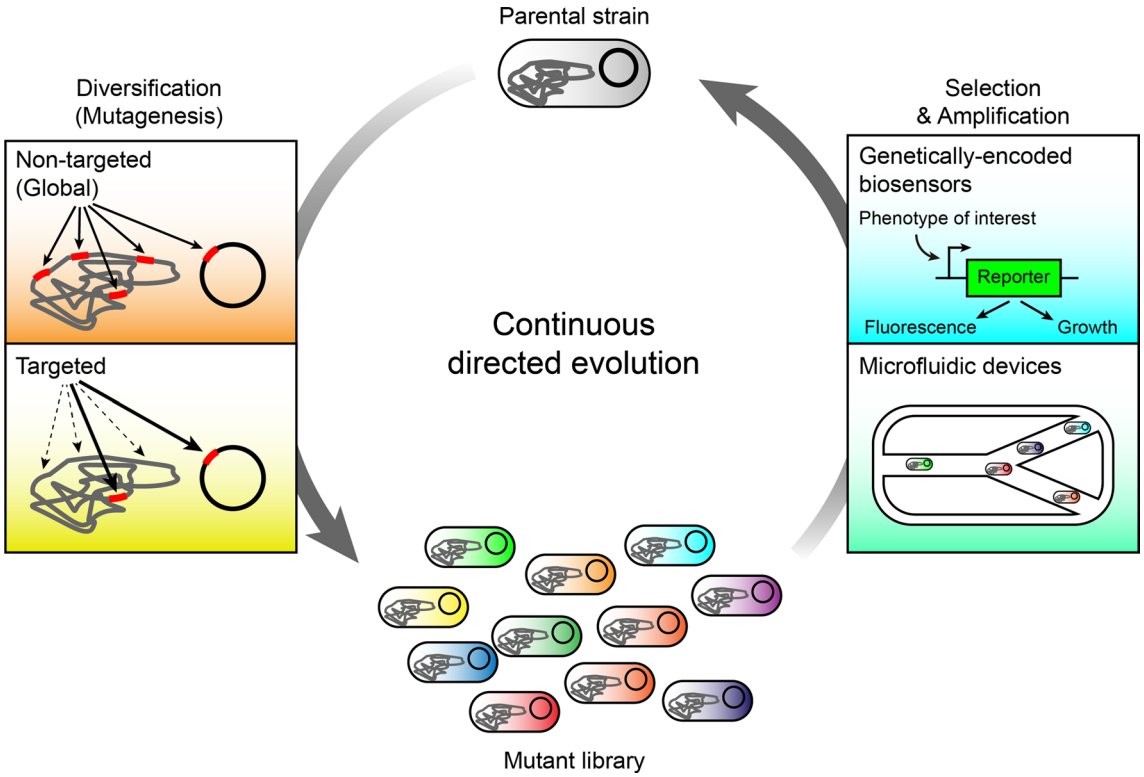


Fig. 1 Scheme of evolutionary engineering

Tools and systems for generating diversity

Directed evolution guides a mutant pool towards desired phenotypes by associating the phenotype with the fitness of a variant in the iterative cycle of diversity generation, selection, and amplification. A concept of the fitness landscape is generally used to illustrate the effect of a genetic variation to a given phenotype of interest. In the fitness landscape, the horizontal plane represents different genetic variations and the magnitude of the phenotype of interest for each genetic variation is plotted as height. Because the fitness landscape resembles a mountain range, we can interpret the goal of evolutionary engineering as climbing up the fitness landscape to reach a maximum peak where the genetic variation at such a point results in the phenotype of interest at its maximum [73]. To successfully evolve biomolecules, genetic pathways, or even genomes in a directed manner, it is crucial that the evolution system meets three fundamental criteria. (i) The mutant library should be designed to contain sufficient genetic diversity to increase the probability of reaching a maximum peak in the fitness landscape [73]. (ii) Appropriate selection pressure should be applied to eliminate inferior mutants, so that propagation of false-positives or cheater mutants is minimized [86, 102]. (iii) The evolution cycle should be easily iterated for efficient evolution of a desired phenotype.

In vitro mutagenesis technologies such as error-prone PCR and DNA shuffling have been broadly utilized for directed evolution [82, 103, 104], as they allow a tunable mutation window in a targeted region. Nevertheless, in vitro mutagenesis is labor-intensive and involves sub-cloning and transformation, which leads to significant loss of the mutant pool because of the limited transformation efficiency [4, 18]. To overcome the drawbacks of in vitro methods and repeat iterative cycles of directed evolution, in vivo approaches have been developed by creating mutagenesis machinery in the cell. However, typical in vivo mutagenesis such as hypermutator strains [29, 30] or mutator plasmids [3, 78] involve the risk of accumulating detrimental mutations in the host genome, because every genetic information in the cell is equally affected by mutagenesis [70]. As random mutagenesis (non-targeted, global) threatens the viability of the host cell and eventually directed evolution, orthogonality, the ability to focus mutations only to a target site (targeted) not to non-target sites (Fig. 2a), has become an integral feature of in vivo mutagenesis while showing a good mutation rate, window, and spectrum. Here, we reviewed several mutagenesis tools (Table 1) according to their strategies to achieve the orthogonality of mutagenesis by (i) implementing an orthogonal cis–trans pair, (ii) detouring the central

dogma, (iii) repurposing a pair of programmed guide RNA and CRISPR-associated endonuclease, and (iv) exploiting the infectious cycle of phage.

Orthogonal pair: ColE1/Pol1, OrthoRep, TaG-TEAM, Muta T7

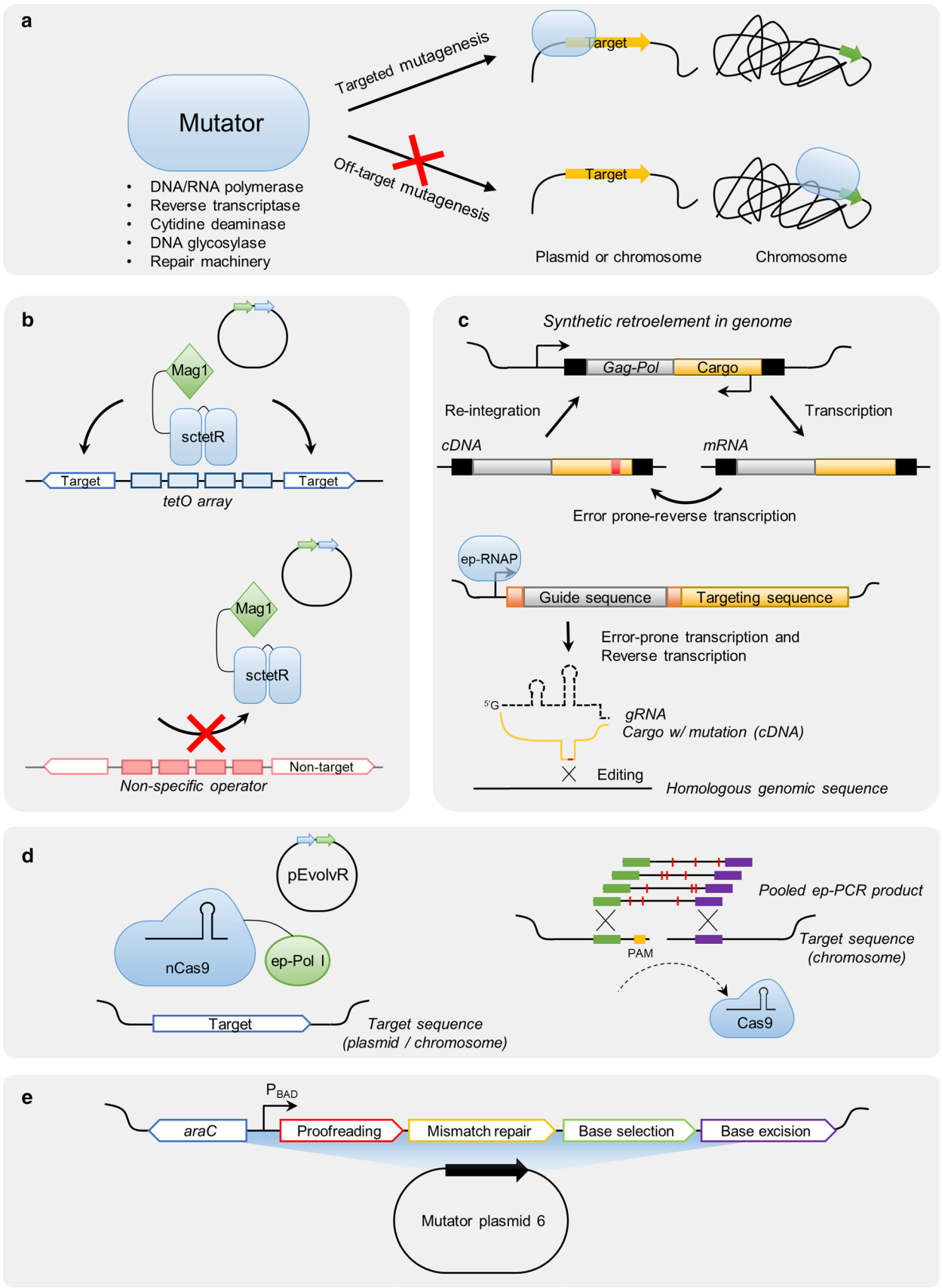
It is pivotal to mutate the target sequence while maintaining the genomic integrity and error-prone capacity of the mutagenesis machinery to sustain directed evolution continuously within the cell. To minimize the interaction of error-generating machinery with unwanted genetic loci, orthogonal replication pairs are employed as in vivo mutagenesis tools.

The ColE1 origin found in bacterial plasmid requires initial transient catalyzation of DNA polymerase I (Pol I) for its distinct replication machinery. Given the complete reliance on Pol I, ColE1 provides the cis element for targeted mutation and amplification [10]. Through catalysis by error-prone Pol I encoded in another plasmid, the gene of interest localized downstream of the ColE1 origin can acquire mutations and replicate simultaneously. Although unwanted chromosomal mutation is inevitable because of the contribution of DNA polymerase I to Okazaki fragment joining and DNA repair [46], mutagenesis performed under saturated growth conditions maximizes the gap between the plasmid and chromosomal mutation rate. Despite the even distribution of mutations, the switch from the error-prone Pol I to the endogenous Pol III during the target plasmid replication limits the mutation window to 650 base pairs (bp) downstream, which has a minimal chance of encoding a single gene.

Another orthogonal pair of plasmid and polymerase is applied in a yeast-specific mutagenesis tool named as OrthoRep [70]. The linear cytoplasmic plasmid pGKL-1 (p1), derived from *Kluyveromyces lactis* used in OrthoRep, necessitates dimerization of the terminal protein (TP) anchored at the terminal end and TP-DNAP for initiation of replication [87]. Deduced from the distinctive protein-primed replication machinery and spatial segregation from the chromosome, Ravikumar et al. hypothesized that p1 could be extended as a vector of the target gene for orthogonal mutagenesis and observed an elevated mutation rate on p1 using error-prone TP-DNAP1 with minimal cross-talk between the host chromosome [69]. Recently, they further engineered TP-DNAP1 to reduce fidelity without impairing processivity, which dramatically increased the mutation rate. Error-prone TP-DNAP1 is an indispensable enzyme for sustaining the replication of p1 and leads to the inevitable introduction of mutations on its pair of plasmids, preventing impairment of the trans element of mutagenesis on the nuclear plasmid. The low off-target mutation rate enables the replication over 90 generations with a stable mutation rate, which is an important property for in vivo mutagenesis to facilitate continuous evolution. However, the applicable host being confined to the yeast strain and meticulous genetic manipulation limits the applications of this method.

Finney-Manchester and Maheshri constructed targeting glycosylases to embedded arrays for mutagenesis (TaG-TEAM) system based on base excision repair (BER) and the specific interaction between tetR and its cognate operator, tetO (Fig. 2b) [26]. 3-Methyladenine DNA glycosylase derived from *Saccharomyces cerevisiae* has a broad substrate range and excises alkylated bases in the first step of BER [62]. Apyrimidinic/apurinic (AP) sites caused by glycosylase are further removed from the phosphate backbone through the action of AP endonuclease. However, a previous study reported that overexpressed glycosylase excises normal base pairs in AP endonuclease knock-outs, increasing the spontaneous mutation rate [96, 97]. This is because unprocessed AP sites cause stalling of DNA synthesis machinery and recruit error-prone trans-lesion polymerase [7]. The authors designed a fusion of non-specific yeast glycosylase Mag1 with single-chain tetR (sctetR). The fusion protein bound to one of the tetO arrays near the mutagenesis target and excised the normal bases nonspecifically. As a result, mutations were observed spanning 10 kb bi-directionally from the tetO array. TaG-TEAM provides a fixed mutation range long enough to contain several genes, albeit the mutation rate declines as the distance from the anchored site increases. Moreover, there are tricky requirements; an array of tetO should be embedded in the vicinity of GOI and DNA repair machinery in the host cell must be partially shut down, which increases the risk non-targeted spontaneous mutations.

Adopting a similar concept as a fusion protein, the Muta T7 system utilized an orthogonal pair of the T7 promoter and cognate polymerase [58]. As opposed to TaG-TEAM which induces mutations around tethered sites, the processivity of T7 RNA polymerase delivers mutations on a sliding template. Thus, Muta T7 can establish a mutation window using the T7 promoter and tandem terminator [75]. Moore et al. selected cytidine deaminase as a mutator to promote the transition of cytosine to uracil, which is recognized as thymine by DNA polymerase. However, the accessible mutational spectrum configured by amino acid substitution from cytidine deaminase is 32% of the total case, which confers mutational bias [31]. To fill this gap, an additional T7 promoter is introduced in the opposite orientation to broaden the mutation spectrum. Nevertheless, A and T remain intact, with the system compromising the unreachable landscape of the genotype.



◂**Fig. 2** In vivo mutagenesis methods. **a** Orthogonality of mutagenesis methods. **b** Targeted mutagenesis based on orthogonal tetO-tetR pair. Mag1-sctetR fusion protein expressed from the plasmid binds tetO array and excises bases on the target locus (upper). In contrast, Mag1-sctetR fusion cannot excise non-target genes located alongside non-specific operators (lower). Mag1, 3-methyladenine glycosylase; sctetR, single chain tetR. **c** Synthetic retroelement constructed using retrotransposon Ty1 and mutagenic T7 RNA polymerase-retron system. Mutagenesis target is cloned into the genome as a genetic cargo between Ty1RT (*Gag-Pol*) and 3′-LTR (Long Terminal Repeat). Transcribed mRNA is converted into mutation-containing cDNA as a result of error-prone reverse transcription by reverse transcriptase and re-integrated into the genome. The red box indicates mutation and the black boxes indicate LTRs (upper). Error-prone T7 RNA polymerase synthesizes the transcript of targeting sequence, generating mutations. Then, the transcript is converted into cDNA. Subsequently, msDNA complex comprised of gRNA and error-containing target cDNA edits homologous genomic locus (lower). Orange box and red line indicates reverse transcriptase recognition site and mutation, respectively. **d** CRISPR-guided mutagenesis tools. Error-prone DNA polymerase I introduces mutations to the target sequence guided by gRNA and nCas9 (left). CRISPR-mediated double-strand break induces homologous recombination with pooled error-prone PCR products (right). Green, purple, and red boxes indicate homologous sequences and mutation, respectively. **e** Mutator plasmid. Genes located downstream of P_{BAD} promoter are overexpressed upon arabinose induction to disrupt replication maintenance and repair pathways

Detour central dogma: ICE (Ty1 retrotransposon), retroelement

A mutagenesis tool implementing reverse transcription (RT) was established to minimize the interaction with the innate cellular machinery of the host that is not equipped with reverse transcriptase. Crook et al. applied the yeast long terminal repeat transposon Ty1 to enable in vivo continuous evolution (ICE) (Fig. 2c, upper) [15]. The target gene inside the retrotransposon cassette as cargo gains mutations in tandem with error-prone transposition. The authors optimized factors such as the cargo expression level, host factors involved in the transposition rate, and induction conditions to increase targeted mutagenesis by over 50-fold compared to the wild type and, therefore, enlarge the library size in each round. Retroelement-based mutagenesis showed a consistent mutation rate across cargo and moderate mutation bias compared to mutagenic polymerases. Interestingly, by comparing two independent in vivo continuous evolution methods, different mutation profiles were found to be induced from the Ty1 cassette conveying xylose isomerase and one xylulokinase variant with different consumption rates as a pair. This supports that the mutation range comprising multiple genes requires a parameter in the mutagenesis tool that drives a novel evolutionary landscape for metabolic engineering. Moreover, by combining the fitness of evolved mutants from ep-PCR and the retrotransposon element, more than half of the latter surpassed the former. Despite the appealing performances of the Ty1 retrotransposon cassette as a mutagenesis tool, the applicable host is limited to yeast strains, which allows Ty1 transposition.

RT-based targeted gene editing machinery operating in prokaryotic taxa was reported, followed by the discovery of retrotransposons in eukaryotes [25]. Based on the previous studies, Simon et al. optimized the editing frequency and length for continuous evolution applications (Fig. 2c, lower) [81]. In contrast to Ty1-based mutagenesis, mutagenic T7 RNA polymerase introduce errors on the transcript of multicopy single-stranded DNA containing a target sequence [55]. Error-containing targeting DNA converted by RT edits the genomic sequence driven by homologous recombination. Simon et al. controlled the promoter strength of the retrotransposon cassette and fidelity of T7 RNA polymerase along with the efficiency of host DNA repair machinery to improve the editing frequency. However, the short mutation range and limited number of multiple point mutations as well as damaged repair machinery prevents successful continuous evolution.

CRISPR guided: EvolvR, CREATE, CasPER

As a navigator of the CRISPR-Cas9 complex, guide RNA designates the target loci based on sequence homology, which confers orthogonality to CRISPR-based mutagenesis tools. A mutagenesis system named as EvolvR utilizes a fusion protein which consists of a nicking variant of Cas9 (nCas9) and error-prone DNA polymerase I (Fig. 2d, left) [31]. nCas9 guided by gRNA forms a nick on targeted loci where coupled DNA polymerase synthesizes a new strand of up to 200 bp with low fidelity and cleaves the displaced strand. Halperin et al. tuned each component to enhance the mutation rate and range and minimize off-target effects. They aimed to alleviate the affinity of nCas9 with DNA to promote mutagenic sliding of Pol I while introducing a triple combination of fidelity-reducing mutations on Pol I. The processivity of Pol I is also modified to expand the mutation window. Finally, the coding sequence of EvolvR is optimized to fit the codon usage and remove innate strong RBS to diminish off-target mutations. In addition, combinatorial mutagenesis within a single target gene and multiplexed targeting on distant genes can use multiple targeting sequences. Considering the average size of the gene, however, the tunable window of EvolvR requires the design and use of several programmed gRNAs simultaneously. Moreover, non-targeted nickase activity also shows a risk of unintended genomic modification.

Another CRISPR-based mutagenesis system coupled with in vitro mutagenesis named as CRISPR-enabled trackable genome engineering (CREATE) was introduced by Garst et al. [28]. CREATE employs homologous repair to integrate a mutation pool by generating site-specific DSBs by programmed gRNA and Cas9 in *Escherichia coli*. As reported

Table 1 Mutagenesis methods

Location	Orthogonality	Name	Machinery	Mutagenesis	Machinery	Mutational range	Host organism	Disadvantage	Reference
In vivo	Orthogonal pair	ColE1/PolI	Orthogonal plasmid/polymerase pair	Error-prone DNAPI	Replication	< 2 kb	*E. coli*	Not fully orthogonal Host cell is limited to *E. coli*	[10]
		OrthoRep	Orthogonal plasmid/polymerase pair	Error-prone TP-DNAPI		22 kb	Yeast *S. cerevisiae*	Difficult genetic manipulation Works only in yeasts	[69, 70]
		Muta T7	Phage derived promoter/polymerase pair	Cytidine deaminase	Repair	Multi-kb		Narrow mutation spectrum (C:G→T:A)	[58]
		TaG-TEAM	tetO/tetR	DNA glycosylase-driven error-prone homologous recombination		20 kb	Eukaryotic	tetO array should be inserted in genome Mutational range is fixed to 20 kb	[26]
	Compartmentalized	PACE	Separation of space (phage-assisted)	Mutator plasmid MP6	Repair deficient		Phage-susceptible	Specialized turbidostat is required	[3, 23]
	Detour central dogma	ICE	Retrotransposon	Error-prone reverse transcriptase	Reverse transcription	5 kb/cargo	Eukaryotes	Efficiency is limited by homologous recombination	[15]
		Retron	Retron	Error-prone RNAP	Transcription	Within 30 bp (11 bp)	Prokaryotes	Mutational range is limited	[81]
	CRISPR-guided	EvolvR	gRNA targeted	Nickase + error-prone DNAPI	Repair	200 bp		Off-target effect	[31]
In vitro		CasPER		DSB + error-prone PCR	Recombination	300–600 bp		Unwanted mutation could be introduced by the competition with NHEJ	[35]
		CREATE		DSB + oligonucleotide synthesis					[28]

previously, a DSB dramatically increases the efficiency of HR by at least two orders of magnitude [76, 83]. They support a web tool for the automated design of a CREATE cassette which serves as a guiding sequence for targeting, template used in HR, and barcode for tracking. However, the toxicity arising from the DSB decreases transformation efficiency, limiting the expandability of library construction and stability of the barcode in plasmids and restricting the tracking of genotype–phenotype relationships. Ronda et al. introduced another CRISPR-based multiplexed genome engineering method called CRISPR/Cas9 and λ Red recombineering-based MAGE (CRMAGE) [74]. This technology

utilized two curable plasmids encoding Cas9, λ Red protein, and target-specific sgRNA under the control of inducible promoters. They also contained self-eliminating genetic circuit for subsequent rounds and efficiency-enhancing elements such as RecX and dam methyltransferase that confers transient MutS and RecA- phenotypes. The CRMAGE system showed higher efficiency compared to the traditional MAGE and was provided with a web-based tool to facilitate the design of an experiment. However, the incomplete killing of non-engineered, wild-type cells remains a challenge. Jakočiūnas et al. developed Cas9-mediated Protein Evolution Reaction (CasPER) which enables the integration of large mutant fragments into genomic loci (Fig. 2d, right). CasPER exploits the specific targeting ability of CRISPR and DSB-induced HR and an error-containing target gene generated by five consecutive ep-PCR [35].

Mutator plasmid

Badran and Liu developed a mutator plasmid as an inducible episomal vector, which prevents redundant replication maintenance mechanisms such as proofreading, mismatch repair, and base selection (Fig. 2e) [3]. By combining several genes representing the mutator phenotype, plasmids with diverse mutational spectra were constructed. Among them, MP6 showed the highest mutation rate and unbiased mutation spectrum along with a very large dynamic range. The mutagenic performance of the random mutagenic plasmid was shown to be sustainable in phage-assisted continuous evolution in a subsequent study [2]. Although partially blocked DNA repair machinery shows compromised viability, the mutation rate does not exceed the error threshold of essential genes [9, 45].

Tools and systems for screening and selection

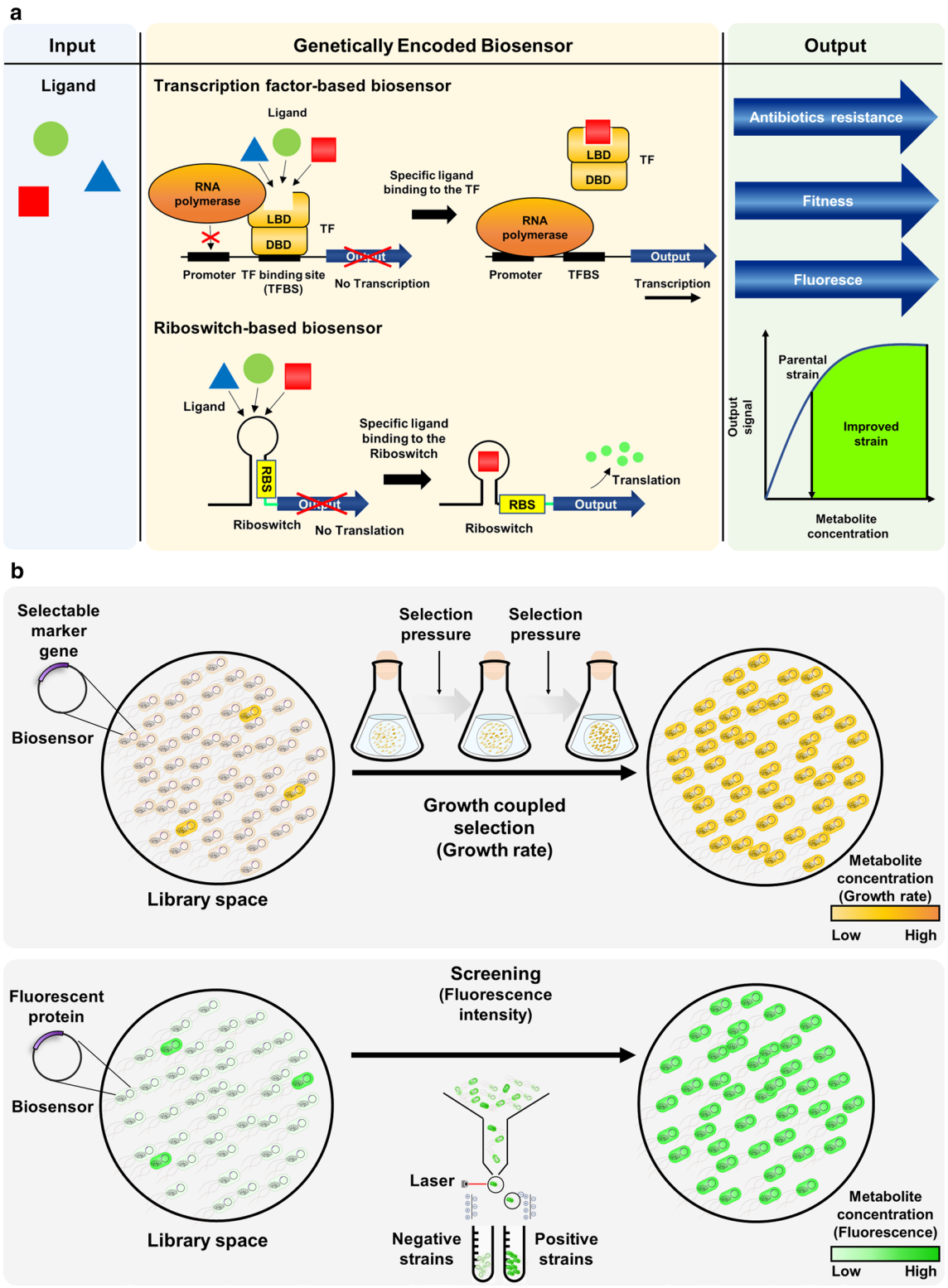
Directed evolution and evolutionary engineering have enabled the production of numerous strains to form new chemical bonds, such as carbon–silicon bonding that has never been observed in nature [44], improve the solubilities of heterologous proteins [53, 93], and increase tolerance to chemicals [5, 11, 41, 47]. The previous studies using these applications have already been reviewed [14, 63]. Thus, here, we focus on artificial selection and screening for chemical high-producing cells using synthetic genetic devices consisting of a biosensor including transcription factor or RNA-based regulatory elements and selectable marker or fluorescence reporters with fluidic devices. It is important to create genetic diversity and effectively screen variants showing a desired phenotype (typically chemical high-producer) from the generated library in directed evolution and adaptive laboratory evolution [18, 90]. As a promising industrial strain, *E. coli* can be used with maximum library sizes of up to 10^{10}; this value is limited by transformation efficiency [33, 42, 91]. However, because the phenotypes are typically inconspicuous, it is difficult to effectively identify the improved strain with the desired phenotype [72]. In the conventional screening methods, individual strains are separated, and their productivities are measured directly by high-performance liquid chromatography, gas chromatography, and microtiter plates. However, the analytical methods are less likely to detect meaningful positive mutants in the library, because the number of variants tested per experiment is generally lower than 10^4, which is time-consuming and labor-intensive for assessing library spaces [51, 52, 97]. Therefore, high-throughput screening methods are required to alleviate the limitations and cover the entire library size to effectively screen for positive mutants [39]. High-throughput screening of strains with phenotypes such as high production of target metabolites has become possible with the introduction of a screening and selection system based on a genetically encoded biosensor including a riboswitch and transcription factor (Fig. 3a, Table 2) [57]. A synthetic genetic device for high-throughput screening consists of a specific upstream ligand-responsive biosensor and downstream expression module [51]. An antibiotic-resistant gene, essential gene, or gene encoding a fluorescent protein is mainly used as an expression module. When a biosensor specifically binds to a cognate ligand, conformational changes occur in response to the intracellular ligand concentration, and expression of the downstream module is regulated at the transcriptional or translational levels [19, 21, 27, 50].

First, we summarize recent studies that have used growth-coupled selection to obtain a strain with an improved target metabolite productivity and review recent studies of cell sorting and screening through fluorescence signal detection based on microfluidics in the following section (Fig. 3b).

Genetic devices and circuits for artificial screening selection

Transcription factor-based selection tools

Transcription factors (TFs) have been reported to regulate expression in response to a variety of metabolites including amino acids, flavonoids, and cofactors [60, 68]. Natural TFs-cognate promoter sets were used as detectors, or artificial promoters that responded to the desired metabolites were identified by proteomics analysis [54]. Selection devices for regulating the expression of antibiotics-resistance genes using TFs as a sensor protein that responds to target metabolites have been constructed and applied [24, 77, 98, 101]. A synthetic selection device responding to the 3-hydroxypropionic acid concentration was constructed to control *tetA* gene



expression exploiting 3-HP-responsive-TF, LysR, and the cognate promoter, P_{C4M}. An aldehyde-binding site library of alpha-ketoglutaric semialdehyde dehydrogenase, a 3-HP production pathway enzyme, was constructed and applied for growth-coupled selection. A selected ALDH variant showed a 2.79-fold improvement in the catalytic efficiency and *E. coli* with an ALDH variant exhibited 25% improved 3-HP productivity compared to the parental strain [80]. Single-cell level quality control devices, population quality control (PopQCs), were developed and applied to prepare

◂**Fig. 3** Genetically encoded biosensors and high-throughput screening methods for metabolite-producing microbial cells. **a** Genetically encoded biosensors. On-type transcription factor (TF)-based biosensor and riboswitch-based biosensor are illustrated. Ligands (particularly, metabolites in this article) bind specifically to their cognate biosensor. Conformational changes occur upon binding of a specific ligand to the ligand-binding domain (LBD) of TF or riboswitch. Both biosensors regulate the expression of 'Output' genes depending on the metabolite concentration. Antibiotic resistance gene, essential gene, and fluorescent protein gene are typically used for the downstream genes in the biosensors. **b** Selection and screening of the chemical producers from a mutant library. Biosensors confer different levels of fitness under the selection pressure or fluorescence to the mutant strains according to the intracellular metabolite concentration. Growth-coupled selection strategy enriches high producers from the mutant population through serial cultures (upper). High-throughput screening analyzes individual cells and collects positive strains using fluorescence-activated cell sorting (FACS) instruments microfluidics devices (not shown in this figure) (lower)

strains producing high levels of chemicals as the major population, while the minor population which could not produce sufficient amounts of metabolites was eliminated by antibiotic selection. In this research, the free fatty acid-responsive transcription factor FadR and synthetic promoter PAR and tyrosine-responsive TF TyrR with the tyrosine-activated promoter, PT1 or PT2, were utilized to maintain the quality of the population for a and b production, respectively [97]. By expanding the scope of evolutionary metabolic engineering to the whole biosynthesis process of metabolites, 18 endogenous loci in *E. coli* were simultaneously mutagenized by multiplex automated genomic engineering within regulatory and coding sequences to construct a mutational library. Expression of the membrane protein TolC was regulated by a natural transcription factor, TtgR, and sensing by the P_{ttga} promoter depends on the naringenin concentration. Toggling positive and negative selection applied to libraries can enrich higher-producing cells and reduce the possibility that cheater cells dominate the population [68]. Modifications of an allosteric transcription factor are based on a computational strategy for altering effector specificity of the protein. In this study, the repressor protein LacI was re-engineered to respond to fucose, gentiobiose, lactitol, and sucralose [85]. In addition to the allostery, the stability of proteins can be used to engineer biosensors. Jester et al. engineered steroid biosensors using dimeric ligand-binding domains, the stability of which can be controlled by the ligand [40]. By fusing each dimeric domain to a DNA-binding domain and a transcriptional activation domain, a full transcriptional activator was able to be reconstituted only in the presence of two ligands that bind and stabilize the dimeric domains. The researchers further engineered the heterodimerization and selectivity of the sensor through directed evolution. Selected mutants were subsequently analyzed by NGS, elucidating the spectrum of mutations that allowed improved sensor performance.

Riboswitch-based selection tools

The riboswitch is a cis-regulatory RNA element that consists of an aptamer domain that specifically binds small molecules and an expression platform that regulates downstream gene expression by causing a conformational change upon ligand binding to the aptamer [39]. The riboswitches used for directed evolution function mainly at two levels: transcriptional and translational. Forming the rho-independent transcription terminator, which regulates gene expression at the transcription level leading to premature transcription termination and sequestering or releasing the ribosome-binding site (RBS), can control gene expression at the translational level [12]. Likes TFs, numerous RNA-based biosensors are known to exist in nature. Furthermore, natural detector elements were identified by methods such as NGS and Parallel Analysis of RNA Conformations Exposed to Ligand binding (PARCEL) [17, 84]. High-throughput screening of the riboregulator can control transcription termination in response to a specific metabolite concentration by combining the transcription start site sequencing and transcription termination site sequencing method based on NGS [17]. Furthermore, an experimental strategy for identifying RNA aptamers in vitro known as PARCEL in the transcriptomes revealed 58 novel RNA aptamers in both untranslated regions and coding sequences [84]. A natural riboswitch was engineered with specificity for non-natural small molecules and no response to the original ligand [20]. For a suitable RNA-based detector that specifically responds to target biochemicals that do not exist in nature, riboswitch binding to the desired chemical can be artificially prepared using an in vitro selection method known as systematic evolution of ligands by exponential enrichment [36, 37, 100]. The riboswitch-based biosensor was successfully applied for growth-coupled selection [61, 88, 89]. For example, a riboselector as a synthetic RNA device that specifically reacts with L-lysine and L-tryptophan to express the tetracycline resistance gene was constructed. The riboselector conferred a fitness advantage to strains with high performance by expressing tetracycline resistance. Particularly, selection of the *ppc* promoter library to obtain the high-producing strain of L-lysine resulted in successful enrichment of the top 3 improved strains to 75% of the population in only four rounds of enrichment cycles [99]. In addition to the riboswitch, metabolite-responsive ribozymes have been exploited to select metabolite high producers. For example, a glmS ribozyme, which responds to the glucosamine 6-phosphate concentration, was combined with the 3′-untranslated region of the cytosine deaminase, FCY1, in *S. cerevisiae* to construct a synthetic suicide riboswitch to screen for an *N*-acetyl glucosamine-overproducing *S. cerevisiae* strain. A glutamine–fructose-6-phosphate transaminase mutant library and haloacid dehalogenase-like phosphatases library were

Table 2 Genetically encoded biosensors for chemicals

Product	Detector	Output	Function	Reference
Naringenin	Transcription factor/cognate promoter pair	Expression of TolC	Growth-coupled selection, counter selection	[68]
Glucaric acid	Transcription factor/cognate promoter pair	Expression of TolC	Growth-coupled selection, counter selection	[68]
Threonine	Transcription factor/cognate promoter pair	Expression of eGFP beta-Galactosidase	Screening of improved production strain	[54]
3-Hydroxypropionic acid	Transcription factor/cognate promoter pair	Expression of TetA	Growth-coupled selection	[80]
Free fatty acid	Transcription factor/cognate promoter pair	Expression of TetA	Growth-coupled selection	[97]
Tyrosine	Transcription factor/cognate promoter pair	Expression of TetA	Growth-coupled selection	[97]
Naringenin	Riboswitch	Expression of GFP	Naringenin sensor	[36]
Lysine	Riboswitch	Expression of TetA	Growth-coupled selection	[99]
N-acetyl glucosamine	Ribozyme	Expression of FCY1	Growth-coupled selection	[49]
Tryptophan	Riboswitch	Expression of sGFP	Representing the intracellular L-tryptophan concentration of each single cell	[38]
Theophylline	Aptazyme	Expression of GFP	Screening of improved enzyme	[56]

efficiently screened with the growth-coupled genetic circuit, resulting in the *N*-acetyl glucosamine overproducer [49].

High-throughput screening with fluidic systems

High-throughput screening, in which positive strains dominate following library selection, has been widely used for directed evolution, as it is possible to exploit selectable markers and appropriate selection pressures without additional equipment. However, cheater cells adapted to resistance under the selection pressure may be selected rather than positive mutants with the desired phenotype, which are not selected. Counterfeit transcription, such as a mutation in a promoter expressing a metabolic sensor or selectable marker in an antibiotic resistance gene, may have growth advantages under the selection pressure regardless of production of the desired metabolite. As a result, cheater cells dominate the population [72]. In addition, to enrich the positive strain, labor-intensive and long-term serial culture is required. Furthermore, artificial selection cannot confirm the target metabolite production ability at the individual cell level in real time [19]. Therefore, genetically encoded biosensors were constructed by connecting expression of the fluorescence markers GFP and RFP to the target metabolite concentration; these biosensors have been applied with various types of analysis equipment such as microfluidics devices and fluorescence-activated cell sorting (FACS) instruments [38, 92]. FACS can be used in combination with genetically encoded biosensors to screen mutant enzymes with increased product selectivity and activity. For example, a caffeine demethylase library containing 10^6–10^7 variants was screened in a few hours through FACS using fluorescence from a ribozyme-based biosensor as a readout. A screened variant showed 22- and 33-fold higher selectivity and activity, respectively, compared to the parental strain. Modular assembly of input domain (aptamer) and output domain (ribozyme) of the biosensors can create synthetic RNA switches that will be applied to various metabolic pathways [56]. Furthermore, FACS was also utilized to select variants with increased target metabolite production. For example, FACS was coupled with a biosensor to select threonine high producers [54]. A threonine biosensor was developed by placing a fluorescent protein gene under the control of a threonine-responsive promoter cysJHp. A mutant library containing 2×10^7 mutants was constructed based on an industrial threonine producer strain ThrH(pTZL2). The mutant library was screened using FACS and the threonine biosensor within 1 week and 465 mutants were selected. Forty-four strains sorted from the library showed higher threonine productivities compared to the parental strain, and the highest mutant produced approximately 18% higher levels of threonine [54]. In addition to FACS, a microfluidics system was applied for the screening of high-performance cells. The microfluidic static droplet array can trap a single *E. coli* cell in a droplet in assigned wells. A tryptophan riboswitch-based biosensor linked to the fluorescence protein was constructed and tryptophan overproduction strains were collected by detecting the output signals with the static droplet array system [38]. Screening that relies on the intracellular concentration of a substance using biosensor neglects the extracellular content. For secreted products, it is difficult to screen for true-positive strains with a system

that detects only intracellular concentrations, and obtaining false-positives is very likely. This resulted in a reduced probability of screening of true-positive strains that produce and secrete more product and increased probability of screening of false-positive strains that accumulate more products. To evaluate extracellular concentrations, screening with a droplet compartment is necessary. A single cell or cells required for co-culture were compartmented into a single droplet, and then incubated for fluorescence-based screening to ensure true-positive strain screening including extracellular content. One study conducted library screening of innately fluorescent riboflavin-producing strains. Single-cell FACS and droplet-based FACS screening were conducted to demonstrate that the extracellular concentration represents total production rather than the intracellular concentration. The results showed that screening based on extracellular concentrations was more effective for isolating strains with higher total production [92].

Systems for continuous evolution

Natural evolution occurs continuously: genetic diversity is generated, selective pressure is applied, and adapted offspring reproduce. This cycle repeats indefinitely. In contrast, many evolutionary engineering techniques involve frequent intervention at each step of evolution. This requirement for intermittent handling by a researcher limits the efficiency, multiplexity, and scalability of evolutionary engineering. Therefore, researchers have attempted to conduct artificial continuous evolution which operates autonomously with minimal intervention [16]. The most critical issue for establishing continuous evolution would be how to control mutagenesis to generate sufficient genetic diversity at the genetic level while maintaining host cell stability. To address this challenge, there have been several attempts to develop genetic circuits for regulating mutagenesis or mutating only desired genetic loci.

First, synthetic genetic circuits enabled controlled mutagenesis in response to the phenotype of interest. In this approach, the expression of mutagenic genes is repressed after achieving the desired phenotype in a cell through the accumulation of sufficient mutations. For example, metabolite-responsive promoters controlled the expression of an error-prone DNA polymerase subunit to evolve mutant strains with improved metabolite productivity such as tyrosine and lycopene [13]. In another example, an engineered riboswitch responsive to changes in pH was utilized to control the expression of an integrase [65]. The pH-dependent expression of the integrase, in turn, controlled the expression of a mutagenic gene. Mutant strains that maintained intracellular pH at a neutral value or in a low pH environment deactivated mutagenic gene expression and stopped further mutagenesis.

Next, mutagenesis can be focused to desired genetic loci to minimize any undesired non-specific mutations that may lead to serious growth retardation or even elimination of the host cell. These strategies are explained in Sects. 2.1, 2.2, and 2.3. The target gene(s) was cloned in a specific plasmid or locus with specialized elements in the OrthoRep [70] or using the retrotransposon method [15], respectively. These methods mutated the cloned "cargo" efficiently, but background mutagenesis was maintained at a low level. In contrast, EvolvR [31] enabled targeted mutagenesis at any locus in the genome by designing gRNAs for target genes. However, this method has a relatively short mutagenesis window (~350 nucleotides) compared to other methods. Another well-known pioneering strategy is using viral replication to propagate the evolved mutants. The phage-assisted continuous evolution (PACE) [23] system utilized the M13 bacteriophage genome as a vector for target genes. In a continuous bioreactor, *E. coli* cells are used to mutate the target gene, and mutants that activate the expression of a phage coat protein are packed in an M13 bacteriophage virion. This released virion infects fresh *E. coli* cells to continue the next round of mutagenesis and selection. Phage-assisted continuous evolution has been utilized to evolve various proteins including TALEN [34], insecticidal toxin [2], aminoacyl-tRNA synthetase [8], RNA polymerase [67], protease [64], antibody [93], and Cas9 [32].

In addition to mutagenesis methods, a continuous culturing apparatus was fabricated to support continuous evolution. Evolutionary engineering requires a continuous culturing system that allows for a multiplexed experiment with highly controlled culture parameters. However, the parameters in batch culture cannot be precisely controlled, while current continuous culture systems are not suitable for parallelized cultures. Recently, a framework for continuous culture known as eVOLVER was reported [94]. This system measures various culture parameters such as optical density or temperature in real time and enables automated control of the parameters. Moreover, a high-throughput evolution study using eVOLVER was conducted for 468 continuous cultures, at a level that cannot be achieved using previous methods. Because of its modularity, small size, and automated nature, multiplexed evolutionary engineering can be performed more easily using this platform.

Concluding remarks

Evolutionary approaches have enabled engineering of biomolecules and microorganisms for useful applications. Discovery of new biological components and mechanisms have led to the development of advanced technologies for

mutagenesis and screening. In this progression, synthetic biology has provided a theoretical and experimental foundation to create synthetic genetic circuits. In addition, microfluidic and millifluidic systems have assisted in cultivation and screening in a high-throughput manner. Furthermore, previously discrete steps in evolutionary engineering can now be merged, allowing for continuous directed evolution without intervention by researchers. Even with this significant progress, some challenges remain. The size of genetic cargo that can be mutated should be larger than the current size limit (~ 20 kb) to evolve large genetic constructs such as biosynthetic gene clusters for secondary metabolites. Universal methods that can be applied to both prokaryotes and eukaryotes are needed for a wide variety of applications. For example, microbial consortium consisting of prokaryotic and eukaryotic cells can be engineered as a whole using such universal method. Finally, evolved genes, pathways, and strains from initial rounds of continuous evolution can be recombined with each other during the evolution process to provide an improved mutant pool for the next round of evolution which will eventually facilitate the identification of superior mutants in a limited timeframe.

Acknowledgements This work was supported by the C1 Gas Refinery Program [Grant Number NRF-2018M3D3A1A01055754] and the Global Research Laboratory Program [Grant Number NRF-2016K1A1A2912829] funded by the Ministry of Science and ICT of the Republic of Korea through the National Research Foundation of Korea and by the Korea Institute of Energy Technology Evaluation and Planning (KETEP) and the Ministry of Trade, Industry & Energy (MOTIE) of the Republic of Korea [Grant Number 20174030201600].

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interests.

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