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MINI-REVIEW

Combinatorial and high-throughput screening approaches for strain engineering

Wenshan Liu · Rongrong Jiang

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Abstract Microbes have long been used in the industry to produce valuable biochemicals. Combinatorial engineering approaches, new strain engineering tools derived from inverse metabolic engineering, have started to attract attention in recent years, including genome shuffling, error-prone DNA polymerase, global transcription machinery engineering (gTME), random knockout/overexpression libraries, ribosome engineering, multiplex automated genome engineering (MAGE), customized optimization of metabolic pathways by combinatorial transcriptional engineering (COMPACTER), and library construction of "tunable intergenic regions" (TIGR). Since combinatorial approaches and high-throughput screening methods are fundamentally interconnected, color/fluorescence-based, growth-based, and biosensor-based high-throughput screening methods have been reviewed. We believe that with the help of metabolic engineering tools and new combinatorial approaches, plus effective high-throughput screening methods, researchers will be able to achieve better results on improving microorganism performance under stress or enhancing biochemical yield.

Keywords Strain engineering · Combinatorial engineering · Transcriptional engineering · High-throughput screening · Global transcription machinery engineering · Multiplex automated genome engineering · Fine tuning of gene expression

W. Liu · R. Jiang (✉)
School of Chemical and Biomedical Engineering, Nanyang Technological University, 62 Nanyang Drive, Singapore 637459, Singapore
e-mail: rrjiang@ntu.edu.sg

Introduction

Microbes have long been used to produce valuable chemicals, such as amino acids, antibiotics, organic acids, alcohols, and etc. Classical strain engineering methods of using mutagens had been widely adopted to optimize production in the twentieth century. During the past two decades, metabolic engineering tools have been employed to introduce synthetic pathways into microbes to produce chemicals such as biofuels and pharmaceuticals (Keasling 2010; Stephanopoulos 2008). However, due to the complexity of metabolic and genetic networks, it is still difficult to achieve ideal productivity.

"Inverse metabolic engineering" was introduced to strain engineering by Bailey et al. (1996), which consisted of three steps: "first, identifying, constructing or calculating a desired phenotype; second, determining the genetic factors or the particular environmental factors conferring that phenotype; third, endowing that phenotype on another strain or organisms by directed genetic or environmental manipulation". Based on the first step, combinatorial engineering approaches have been developed to improve the productivity of various chemicals, including biofuels (Alper et al. 2006), natural products (Ozaydin et al. 2013), chemicals (Klein-Marcuschamer et al. 2009), as well as microbial tolerance toward various stresses (Wang et al. 2012b; Zhang et al. 2012b). Various "-omics" tools have been developed to facilitate the determination of factors for the second step (Bro and Nielsen 2004). Further improvement can be achieved by fine tuning of gene expression, such as customized optimization of metabolic pathways by combinatorial transcriptional engineering (COMPACTER) (Du et al. 2012) and multiplex automated genome engineering (MAGE) (Wang et al. 2009b). As shown in Fig. 1, this review will focus on (i) combinatorial approaches on generating genetic diversity, (ii) combinatorial fine tuning of gene expression, and (iii) high-throughput screening methods for mutant identification.

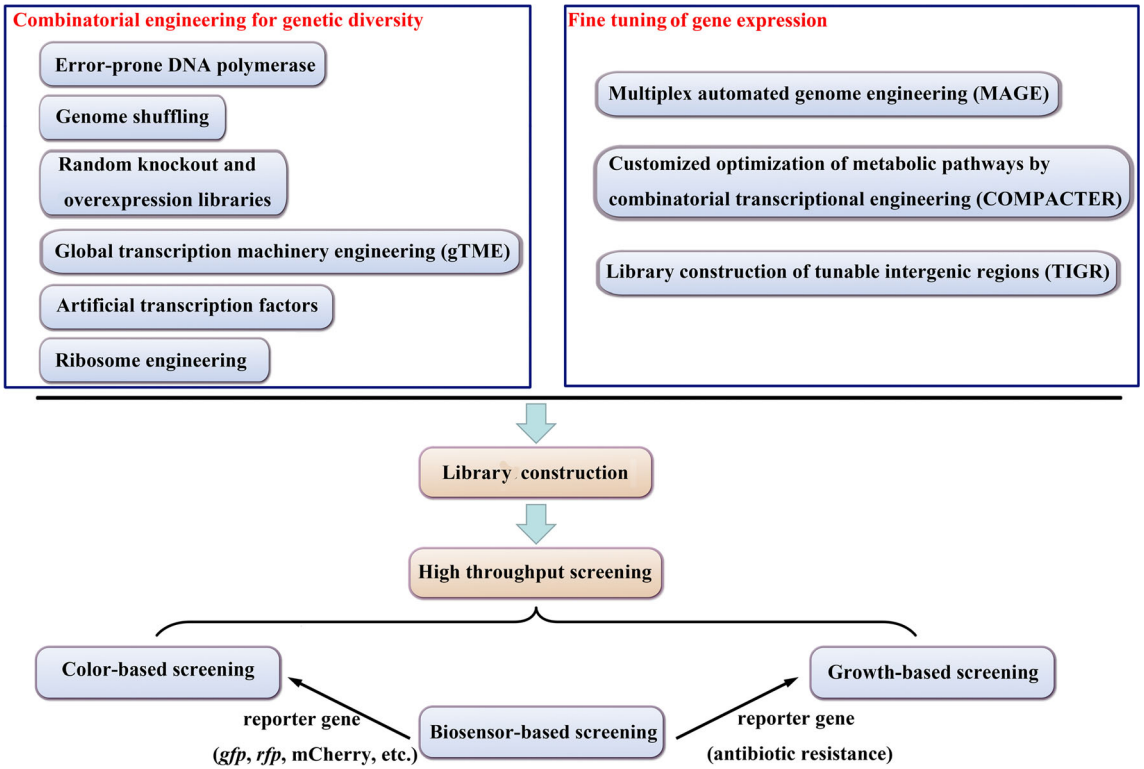

Fig. 1 An overview of combinatorial engineering and high-throughput screening methods for strain optimization

Combinatorial approaches to generate genetic diversity

Combinatorial approaches are developed to engineer microorganisms when there is no detailed genotype-phenotype information available. These methods can help generate genetic diversity in organisms through (i) genome-wide mutagenesis methods, such as the use of error-prone DNA polymerase, and (ii) random mutagenesis of genes or alteration of gene expression, including random knockout/overexpression libraries, global transcription machinery engineering (gTME), and artificial transcription factor engineering. "These methods are combinatorial in nature, that is, they are based on generating genetic diversity in a population followed by screening and selection for improved phenotypes" (Santos and Stephanopoulos 2008).

Error-prone DNA polymerase

An error-prone DNA polymerase with decreased or defective proofreading activity can be generated by mutating its subunits—the proofreading activity of the DNA polymerase subunit is responsible for excising mismatched nucleotides, which is important for keeping high fidelity during DNA replication. Due to uncorrected replication errors generated by error-prone DNA polymerase, random mutations in genome DNA may occur during cell growth, which can accelerate strain evolution process (Miller 1996). Different error-prone DNA polymerases derived from *Escherichia coli* DNA polymerases I and III or yeast DNA polymerase δ have been engineered to alter cell phenotypes (Table 1). For instance, *HpPOL3*, the catalytic subunit of DNA polymerase of *Hansenula polymorpha*, was mutated in its conserved amino

Table 1 Error-prone DNA polymerase approaches for strain engineering

Target/strain	Phenotype	Reference
DNA polymerases I and III		
E. coli	TEM-1 β-lactamase	Camps et al. (2003)
E. coli	T7 RNA polymerase	Esvelt et al. (2011)
E. coli	Growth	Loh et al. (2010)
Bradyrhizobium japonicum	Nitrous oxide reductase activity	Itakura et al. (2008)
DNA polymerase δ		
S. cerevisiae	Thermotolerance	Shimoda et al. (2006)
S. cerevisiae	Ethanol tolerance	Abe et al. (2009a)
S. cerevisiae *Schizosaccharomyces pombe*	N-glycan tolerance	Abe et al. (2009b)
Sc. pombe	Galactose metabolism	Matsuzawa et al. (2011)
Ashbya gossypii	Riboflavin production	Park et al. (2011)
H. polymorpha	Xylose metabolism	Kim et al. (2013)

acid sequence for 3′→5′ proofreading exonuclease activity. The resulting *HpPOL3** was integrated into microbe chromosome, generated a 50-fold higher mutation frequency as compared to the wild type, and resulted in 74 % increase in *H. polymorpha* cell density (Kim et al. 2013). dnaQ is a subunit of DNA polymerase III in *E. coli*, which provides proofreading activity. *E. coli* dnaQ mutator strain was able to survive under much higher antibiotics concentration than the wild type (Tanabe et al. 1999). Moreover, DNA polymerase δ is often targeted to improve eukaryote performance. Shiwa et al. had compared the effects of error-prone DNA polymerase δ with classical mutagen ethyl methanesulfonate (EMS) in *Saccharomyces cerevisiae*. As it turned out, error-prone DNA polymerase exhibited seven times higher mutation frequency than EMS, with even broader diversity in mutation patterns (Shiwa et al. 2012). Further investigations involving genome sequencing can help identify potential mutations that may contribute to the desired phenotype.

Genome shuffling

Genome shuffling allows genome-wide gene recombination of multiple parental strains from different species, using a recursive protoplast fusion method. It can generate progeny with improved or combined phenotype, for instance, combining high productivity and enhanced tolerance in one strain has been proven to be a time-saving and effective method for strain improvement, especially for industrial strain optimization (Zhang et al. 2002). For instance, in order to improve daptomycin production in *Streptomyces roseosporus*, UV and NTG mutagens were used to generate improved parental strains. The daptomycin production increased steadily with each round of genome shuffling via recursive protoplast fusion. After four rounds of genome shuffling, a high-production *S. roseosporus* strain (582 mg/L) had been identified as a potential industrial production strain (Yu et al. 2014). Similar genome shuffling examples could be found in two recent reviews (Biot-Pelletier and Martin 2014; Gong et al. 2009). In summary, genome shuffling has been successfully applied in fungi such as *Aspergillus niger* (Li et al. 2014), yeast such as *S. cerevisiae* (Zheng et al. 2014), or Gram-positive bacteria such as *Streptomyces actuosus* (Wang et al. 2014b). Nevertheless, as for Gram-negative bacteria such as *E. coli*, protoplast fusion may only happen at very low frequency (0.05–0.7 %), due to microbial outer membrane structure (Dai et al. 2005). Moreover, protoplast fusion may lead to less stable recombinants and these progenies can revert to parental phenotypes over time, which will result in inefficient genome shuffling (Hotchkiss and Gabor 1980). To overcome this problem, a new method involving fertility plasmid F was invented in *E. coli* (Winkler and Kao 2012). After the high-frequency recombination strain was generated from the integration of F plasmid into microbe's genome, it could efficiently transfer DNA to a recipient strain via a mating bridge rather than protoplast fusion. The deletion of *traS* and *traT* would decrease cellular surface exclusion and enhance mating frequency and DNA transfer rate significantly. This approach allows continuous in situ recombination in liquid culture and the developed strains are genetically stable.

Random knockout and overexpression libraries

As there is only limited metabolic and genetic network information for microorganisms available, random knockout and overexpression libraries have been developed for strain engineering. Random knockout libraries are often constructed by transposon mutagenesis. The libraries have been adopted to identify negative gene targets of lycopene production in *E. coli* (Alper et al. 2005), riboflavin making in *Bacillus subtilis* (Tannler et al. 2008), poly-3-hydroxybutyrate synthesis in *Synechocystis* sp. PCC6803 (Tyo et al. 2009), and isoprenoid production in *S. cerevisiae* (Ozaydin et al. 2013). Overexpression libraries were achieved by the construction of genomic libraries and have been used to identify positive gene targets, which can improve desired phenotypes such as ethanol tolerance of *S. cerevisiae* (Hong et al. 2010a), lycopene production in *E. coli* (Jin and Stephanopoulos 2007), and acid tolerance of *Clostridium acetobutylicum* (Borden et al. 2010). The drawback of these knockout and overexpression libraries is that they can only reveal the influence of a handful of genes involved in strain phenotype change.

In order to overcome this, a new approach that is based on classical overexpression library and uses microarray analysis has been developed, multiscalar analysis of library enrichments (SCALEs) (Lynch et al. 2007). Three to five libraries of specific genomic fragment insert size (e.g., 1, 2, 4 kbp…) were constructed, and the transformants were mixed and cultured under certain selection pressure. The plasmids of isolated mutants were then extracted for hybridization in a microarray. Since these mutants could grow well under stress and produce a large amount of plasmids, strong signals would be detected from them. As such, the relationship between individual/multiple genes and strain phenotype improvement could be revealed. SCALEs have been used to search for gene targets to improve different phenotypes (Table 2). As SCALEs can only detect population dynamics, it has only been adopted to study growth-related phenotype improvement such as strain tolerance and substrate utilization during fermentation.

To study complex gene interactions, coexisting/coexpressing genomic libraries (CoGeLs) have been developed (Nicolaou et al. 2011), with two coexisting overexpression libraries created from two compatible plasmids containing genomic fragments. CoGeLs have been adopted to search for interactive gene pairs for ethanol/acid tolerance improvement (Nicolaou et al. 2011, 2012).

Table 2 SCALEs applications

Strain	Phenotype	Reference
E. coli	Tolerance to aspartic acid antimetabolites	Bonomo et al. (2008)
E. coli	3-Hydroxypropionic acid tolerance	Warnecke et al. (2010), Warnecke et al. (2008)
E. coli	Growth	Singh et al. (2009)
E. coli	Cryptic aminoglycoside resistance	Struble and Gill (2009)
E. coli	Acetate tolerance	Sandoval et al. (2011)
E. coli	Ethanol tolerance and production	Woodruff et al. (2013a, b)
E. coli	Furfural tolerance	Glebes et al. (2014)

Global transcription machinery engineering

To obtain wider genetic diversity, i.e., to change the expression of hundreds of genes simultaneously, researchers have turned their attention to gTME, which targets the components of transcriptional machinery such as general/global transcription factors. gTME can up- or downregulate hundreds of genes at the same time by applying directed evolution methods on certain components of transcriptional machinery, especially those involved in DNA recognition such as general/global transcription factors and RNA polymerase subunits (Fig. 2) (Alper et al. 2006). Table 3 summarizes all endogenous transcriptional machinery components that have been reported so far. On top of these native regulators, a heterologous global regulator IrrE from *Deinococcus radiodurans* was introduced into *E. coli* to enhance its resistance against ethanol, butanol, and acetate stress (Chen et al. 2011). Similarly,

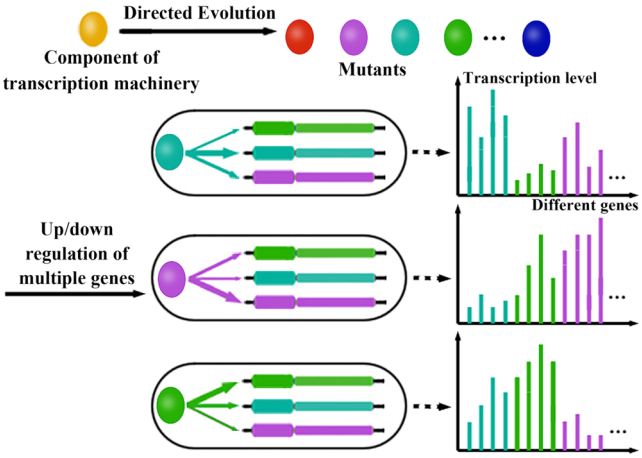

Fig. 2 Scheme of global transcription machinery engineering (gTME). The component of transcription machinery such as global transcription factor is modified by random mutagenesis methods and the mutant library is subjected to selection pressure. The modification to a certain component may result in cell transcription profile changes (*thick arrow* means upregulation, and *thin arrow* means downregulation) (Alper et al. 2006; Chong et al. 2014)

exogenous sigma factor σ^{HrdB} from *Actinoplanes missouriensis*, *Micromonospora aurantiaca*, and *Salinispora arenicola* was selected for random mutagenesis/DNA shuffling and introduced into *Actinoplanes teichomyceticus* to improve teicoplanin production. The best strain was reported to produce 5.3 mg/mL teicoplanin, more than 2-fold of the starting strain (Wang et al. 2014a).

Genome-wide DNA microarray assay has revealed that simple mutations in a transcription factor may lead to cellular transcriptional profile change. For instance, RpoD (RNA polymerase submit σ^{70}) ethanol-tolerant mutant generated via error-prone PCR resulted in the differential expression of 125 genes across *E. coli* genome in the absence of ethanol (Alper and Stephanopoulos 2007).

As compared to classical strain engineering methods of using UV or chemical mutagens, gTME can greatly shorten mutant selection period from months/years to just a few days. The mutant performance has been proven to be comparable or better than using classical approaches (Basak and Jiang 2012; Klein-Marcuschamer and Stephanopoulos 2008; Zhang et al. 2012b). In addition, the winner mutant may possess multiple altered phenotypes at the same time. For instance, the best oxidative stress-tolerant *E. coli* mutant OM3 obtained via cAMP receptor protein (CRP) transcriptional engineering also demonstrated enhanced thermotolerance at 48 °C and organic solvent tolerance, suggesting an overlap in microbial stress defense system (Basak and Jiang 2012).

Artificial transcription factors

Artificial zinc-finger transcription factor (ZFP-TF) has also been reported to reprogram cellular transcriptome and alter strain phenotypes (Park et al. 2003). A natural ZFP-TF normally contains several zinc-finger motifs, each recognizing/binding to a 3-bp DNA sequence; thus, a DNA-binding sequence consisting of several motifs can be built as an artificial transcription factor. Mutant libraries are constructed by randomly assembling 3~4 motifs from dozens of available motifs, which can bind to genome DNA and change gene expression (Park et al. 2003). This method has been used to improve *S. cerevisiae* tolerance toward high temperature, osmotic stress, and an antifungal drug ketoconazole (Park et al. 2003), and *E. coli* resistance against butanol, high temperature, osmotic stress, and cold shock (Lee et al. 2008, 2011; Park et al. 2005). ZFP-TF has also been engineered to improve the growth of a temperature-sensitive strain *S. cerevisiae* $sec14^{ts}$ at restrictive temperature. Relevant gene *NTE1* (serine esterase, homolog of human neuropathy target esterase) was identified via this approach, which was not accessible by classical evolutionary methods (Lee et al. 2013b).

The complexity of library construction for ZFP-TF and global transcription factor is different—gTME library is created via simple error-prone PCR or DNA shuffling, whereas

Table 3 gTME examples

Strain	Transcription factor	Phenotype	Reference
L. plantarum	RpoD	Tolerance toward lactic acid and low pH	Klein-Marcuschamer and Stephanopoulos (2008)
E. coli	RpoA	Butyrate tolerance	Klein-Marcuschamer and Stephanopoulos (2010)
E. coli	RpoA	Butanol tolerance; L-tyrosine production; hyaluronic acid production	Klein-Marcuschamer et al. (2009)
E. coli	RpoD	Ethanol tolerance; lycopene production; surfactant tolerance	Alper and Stephanopoulos (2007)
E. coli	RpoD	Hyaluronic acid production	Yu et al. (2008)
E. coli	RpoS	Hyaluronic acid production	Yu et al. (2008)
E. coli	CRP	Organic solvent tolerance	Basak et al. (2012)
E. coli	CRP	Oxidative stress tolerance	Basak and Jiang (2012)
E. coli	CRP	Ethanol tolerance	Chong et al. (2013a)
E. coli	CRP	Isobutanol tolerance	Chong et al. (2014)
E. coli	CRP	Acetate tolerance	Chong et al. (2013b)
E. coli	CRP	Butanol tolerance	Zhang et al. (2012c)
E. coli	CRP	Osmotic stress tolerance	Zhang et al. (2012b)
E. coli	CRP	Low pH tolerance	Basak et al. (2014)
E. coli	H-NS	Biofilm formation and dispersal	Hong et al. (2010c)
E. coli	Hha	Biofilm formation and dispersal	Hong et al. (2010b)
S. cerevisiae	Spt15/Taf25	Ethanol tolerance	Alper et al. (2006)
S. cerevisiae	Spt15/Taf25	Oxidative stress tolerance	Zhao et al. (2014)
S. cerevisiae	Spt15	Xylose utilization and tolerance	Liu et al. (2010)
S. cerevisiae	Spt15	Xylose metabolism, tolerance, and adaptation to lignocellulosic hydrolysates	Liu et al. (2011)

ZFP-TF library is constructed through the assembly of 3~4 zinc-finger motifs that randomly picked from zinc-finger motifs and the experiments involve rounds of digestion and ligation (Kang and Kim 2000).

Ribosome engineering

Antibiotics such as streptomycin, gentamicin, kanamycin, and chloramphenicol can bind to ribosomes and repress translation. After treatment with these antibiotics, survival variants may have random mutations in their ribosome or RNA polymerase components, which can alter gene expression at translation level. This strategy has been used to optimize antibiotics or enzyme overproduction in bacteria since the last century (Ochi 2007; Ochi et al. 2004) including α-amylase and protease (Ochi et al. 2004), oligomycin, erythromycin (Tanaka et al. 2009), actinomycin (Wang et al. 2009a), and piperidamycin (Hosaka et al. 2009). The best variant selected from the library using antibiotic A can be further improved by another round of ribosome engineering using antibiotic B (Ochi et al. 2004). Ribosome engineering has not only been applied to improve antibiotics production in *Streptomyces* (Table 4), but also used in finding new antibiotics in *Streptomyces*. It was reported that cryptic/silent secondary metabolite biosynthetic gene clusters in *Streptomyces* could be activated by ribosome engineering and, hence, generate new antibiotics/metabolites, even for the strains that could not produce antibiotics originally (Hosaka et al. 2009; Tanaka et al. 2013). Since this approach is similar to spontaneous adaptation in a way, the shortcoming could be its low mutation frequency.

Combinatorial fine tuning of gene expression

Fine tuning of gene expression is crucial for a specific pathway to achieve balanced state. Without such coordination, metabolic imbalance may result in the accumulation of toxic gene products and intermediates or growth inhibition (Glick 1995; Zelcbuch et al. 2013). The key to achieve this balanced state is to regulate each gene expression level in a given pathway, which can also be realized by combinatorial methods, such as MAGE (Wang et al. 2009b), COMPACTER (Du et al.

Table 4 Ribosome engineering examples

Strain	Phenotype	Reference
Streptomyces coelicolor	Actinorhodin production	Wang et al. (2008), Wang et al. (2009a)
Streptomyces avermitilis and *Saccharopolyspora erythraea*	Oligomycin and erythromycin production	Tanaka et al. (2009)
Streptomyces roseosporus	Daptomycin production	Li et al. (2013)
Streptomyces viridochromogenes	Xylanase production	Liu et al. (2013)
Streptomyces actuosus	Nosiheptide production	Wang et al. (2014b)
Klebsiella variicola	Ethanol tolerance and production	Suzuki et al. (2015)

2012), and library construction of "tunable intergenic regions" (TIGR) (Pfleger et al. 2006).

Multiplex automated genome engineering

MAGE was developed to replace multiple sites in genome DNA by desired sequences (Fig. 3a) (Wang et al. 2009b). Synthetic oligonucleotides that contain homologous regions at both ends and DNA replacement fragment at the center can be used to substitute specific DNA sequences in *E. coli* genome during DNA replication, mediated by bacteriophage λ-Red ssDNA-binding protein β. A web-based oligonucleotide design tool has been developed recently to facilitate MAGE (Bonde et al. 2014). Up to 80 sites can be modified or replaced simultaneously by introducing coselective antibiotic resistance markers (coselection MAGE, CoS-MAGE) (Carr et al. 2012). MAGE has been applied for fine tuning of gene expression in two different ways, i.e., promoter replacement and ribosome-binding site (RBS) sequence replacement. For the first, it had been adopted to replace the original promoters of 12 genes in indole pathway with 1~12 T7 promoters. The best modified strain had 4-fold higher indigo yield as compared to the original strain (Wang et al. 2012a). For the second, it was employed to replace the original RBS sequences through degenerated RBS-containing oligonucleotides, which has been applied to 20 genes involved in lycopene biosynthesis. A center region of up to 30 bp in a 90-mer oligonucleotide was designed with degenerate oligonucleotides and a library with total complexity of 4.7×10^5 was constructed, and a 5-fold increase in lycopene yield was achieved (Wang et al. 2009b). Compared with other random RBS replacement methods, such as BioBrick standard assembly-based RBSs replacement (Zelcbuch et al. 2013), MAGE provides a much simpler alternative and can generate a much larger mutant library than BioBrick, which probably could be used to fine tune multiple genes (>20) in complicated pathways.

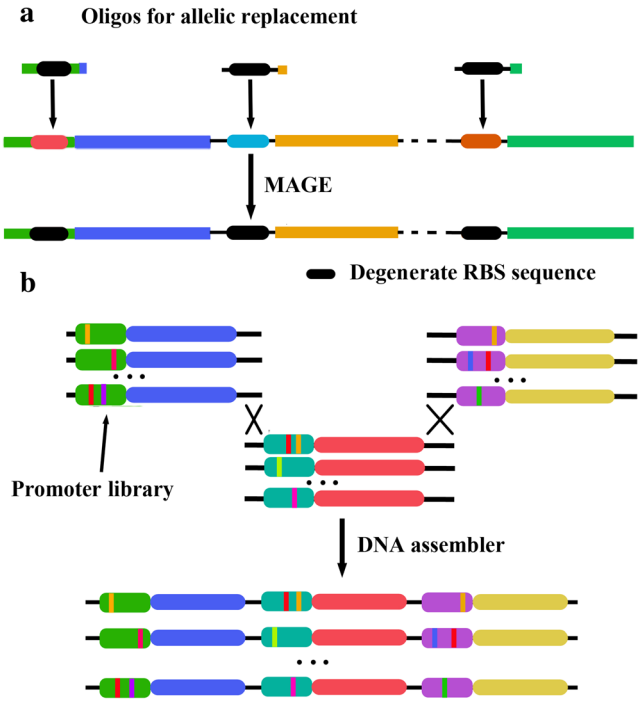

Fig. 3 MAGE and COMPACTER. **a** Multiplex automated genome engineering (MAGE) uses synthetic degenerate RBSs (*black*) to replace original RBSs. The *colorful ends* represent homologous sequences of the desired insertion sites in genome (Wang et al. 2009b). **b** Customized optimization of metabolic pathways by combinatorial transcriptional engineering (COMPACTER) for the fine tuning of a target pathway. The library is created by the assembly of pathway genes with promoters of different strengths (Du et al. 2012)

Customized optimization of metabolic pathways by combinatorial transcriptional engineering

In eukaryotic microbe such as *S. cerevisiae*, promoter engineering allows the manipulation of gene expression at transcriptional level. Most of the promoter engineering studies have used random mutagenesis methods to get a series of promoters with different strengths (Santos and Stephanopoulos 2008). In order to simultaneously tune the expression of multiple genes, "customized optimization of metabolic pathways by combinatorial transcriptional engineering (COMPACTER)" was developed in *S. cerevisiae* (Du et al. 2012), which could simultaneously modify the promoters of multiple genes in a given pathway via DNA assembler (Fig. 3b). COMPACTER was applied to three enzymes in

a xylose-utilizing pathway and two enzymes in a cellobiose-utilizing pathway in *S. cerevisiae*. The engineered strain had 2.1-fold faster cellobiose consumption rate and 2.3-fold higher ethanol yield. COMPACTER could be a useful method to regulate the expression of a few key enzymes in a given pathway, but when the number of enzymes increases, the DNA assembly efficiency may drop.

Library construction of tunable intergenic regions

In addition to aforementioned methods on transcriptional or translational level, libraries of TIGR were constructed for fine tuning on posttranscriptional level. The TIGR was constructed by inserting a RNase cleavage site and two random hairpins into intergenic regions (Pfleger et al. 2006), which can eliminate the interference between genes and affect mRNA stability or translation initiation. For instance, mevalonate production in *E. coli* was optimized by TIGR through constructing TIGR libraries of three genes from mevalonate pathway. A 7-fold increase in mevalonate yield was achieved.

High-throughput screening

One key factor to the success of using these combinatorial approaches is to select an effective screening method. Liquid/gas chromatography-based screening is often time-consuming, whereas agar plate-based screening and fluorescence-activated cell sorting (FACS) can serve as convenient high-throughput screening methods. In general, there are three categories in high-throughput screening: (i) color/fluorescence-based, (ii) growth-based, and (iii) biosensor-based.

Color/fluorescence-based screening

The color/fluorescence-based screening has been mainly used for productivity screening. The simplest screening is that the product itself is pigmented or fluorescent. The shade of color of/around single colony represents the yield of the desired product. Assisted with modern robotics, upward of 10^6 variants can be screened per experiment (Dietrich et al. 2010). Chemicals such as lycopene (Alper et al. 2005), β-carotene (Ozaydin et al. 2013), and astaxanthin (Zelcbuch et al. 2013) belong to this category. An alternative is to use microtiter plate-based screening. A single colony is grown in microtiter plates and can be easily associated with photometric assays to determine product yield. The throughput is approximately 10^5 variants per experiment (Dietrich et al. 2010). Especially, for intracellular florescence, FACS can be used to sort cells according to their fluorescence intensity. Cells exhibiting the highest fluorescence intensity can be obtained instantly and used for further screening. The throughput of FACS is up to 10^9 variants per experiment (Dietrich et al. 2010).

If products are colorless or lack fluorescence, alternatives could be (i) using exogenously added reagents that can react with products to generate color/fluorescence. For instance, nile red and BODIPY (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene) can react with poly-3-hydroxybutyrate to generate fluorescence and be screened by FACS (Lee et al. 2013a; Tyo et al. 2009); (ii) introducing enzymes to convert products into colored/fluorescent chemicals. For example, tyrosinase was introduced to convert L-tyrosine to black pigment melanin for improved L-tyrosine production screening in *E. coli* (Klein-Marcuschamer et al. 2009).

Growth-based screening

Growth-based screening can be employed to select variants with resistance against toxic chemicals, tolerance toward harmful environmental conditions such as organic solvents, acids, or high substrate-utilizing rate (Alper et al. 2006; Basak et al. 2014; Liu et al. 2010). The common way is to culture a large population of transformants in liquid media under certain stress. Taking ethanol tolerance for instance, the transformants are grown under a high concentration of ethanol. Cells with high tolerance toward ethanol stress will survive and grow faster, while those with low resistance will either die or demonstrate growth inhibition (Chong et al. 2013a). This process can be repeated for several rounds to enrich cells with the best tolerance. At last, cell culture will be spread on agar plates and single colonies can be picked for verification. This sort of cell culture can also be used in the microarray assay of SCALEs (Lynch et al. 2007).

Another growth-based screening is to employ an auxotrophic strain as reporter strain for high yield strain selection (Chalova et al. 2007; Kim et al. 2010; Pfleger et al. 2007). For instance, to identify a high mevalonate-producing strain, an isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) auxotrophic reporter strain with green fluorescent protein (GFP) was constructed. A new synthetic pathway that produced IPP and DMAPP from mevalonate was then introduced to this reporter strain. Cells were removed by centrifugation after mevalonate-producing strain had been cultured for 24 h, and mevalonate-containing supernatant was used as substrate for the reporter strain. Mevalonate yield was thus reflected by the fluorescence intensity of the reporter strain (Pfleger et al. 2007). Additionally, a computation-based rational design of auxotrophy-dependent reporter *E. coli* strains has been developed recently (Tepper and Shlomi 2011).

Biosensor-based screening

Among microbe-produced chemicals, only a few can be directly screened by color/fluorescence-based screening methods as described above. Many other chemicals are not natively chromophoric or fluorescent and hard to be transformed. As some of these chemicals can bind to RNAs, DNAs, or proteins, biosensor-based screening methods have been developed to sense products of interest and switch on reporter gene expression for screening purposes (Dietrich et al. 2010). Two in vivo genetic biosensors have been reported so far, one is RNA aptamer/ligand-based, and the other is transcription factor-based. The first comes from the interaction between RNA aptamer and ligand. The aptamer can be inserted into the 5′ untranslated region (5′ UTR) as a riboswitch, and the desired biochemical can bind as ligand to this aptmer and induce reporter gene expression. One successful application is the optimization of L-lysine production in *E. coli* (Yang et al. 2013). Products, such as theophylline, can also bind to aptamers and inactivate ribozymes and enhance reporter gene expression (Michener and Smolke 2012).

In a transcription factor-based biosensor system, a transcription factor can bind to a chemical product and then attach to (or release from) a specific DNA site. Different promoters with this specific DNA site can be constructed to switch on the expression of downstream reporter gene when the chemical product concentration reaches a certain level. The specificity, dynamic range, and linear detection range of these biosensors are determined by transcription factor and promoter construction, which needs to be optimized (Siedler et al. 2013; Zhang et al. 2012a). This system has been used to sense succinate/adipate (Dietrich et al. 2013) and flavonoids (Siedler et al. 2013) and screen for improved production of 1-butanol (Dietrich et al. 2013), triacetic acid lactone (Tang et al. 2013), and mevalonate (Tang and Cirino 2011) in *E. coli*, and also the production of L-methionine, L-lysine, and branched chain amino acids in *Corynebacterium glutamicum* (Binder et al. 2013; Mustafi et al. 2012). There are two different types of reporter genes: (i) fluorescent and luminescent genes such as *gfp* and (ii) antibiotic resistance genes such as tetracycline resistance gene (*tetA*), which can be used for tolerance screening. These two kinds of reporter genes can also be used in combination, e.g., *tetA-gfp* fusion gene (Yang et al. 2013).

In addition to combinatorial approaches, high-throughput screening methods can also be an indispensible part for rational strain improvement, which requires controllable and precise gene expression. Different modules around structure genes were engineered for this purpose. The common strategy is to generate a library for a specific module and use a reporter gene such as *gfp*, *rfp*, and mCherry to evaluate the expression level. A typical example is promoter engineering. A promoter library was constructed by mutagenesis of the GAP promoter in *Pichia pastoris*. Yeast-enhanced green fluorescent protein (yEGFP) was the reporter, and 33 mutants of different promoter activity were screened according to their fluorescent (Qin et al. 2011). Similarly, this was also applied to design other elements for gene expression, such as 5′ UTR −10 box and −35 box (Mutalik et al. 2013), RBS and the sequences around RBS (Seo et al. 2013), and small RNA (Na et al. 2013). The prerequisite for these applications is the availability of genetic and metabolic information to determine gene expression level.

Conclusions

The combinatorial strategies and high-throughput screening methods are fundamentally interconnected—a successful high-throughput screening will only be possible if a large starting population with good genetic diversity is applied. Among the combinatorial approaches discussed earlier, genome shuffling and ribosome engineering are likely preferred if the target microorganism lacks genetic tools, whereas error-prone DNA polymerase and gTME are relatively simple to apply when genetic tools are available. Different approaches can be combined together for synergistic effects. For instance, knockout/overexpression libraries were combined with gTME for lycopene production improvement (Alper and Stephanopoulos 2007; Jin and Stephanopoulos 2007). Fine tuning methods can be adopted to further improve strain performance. As for improving strain tolerance or substrate uptaking, growth-based high-throughput screening methods are often employed. On the other hand, color/fluorescence-based screening may be a good option to enhance product yield when target products are natively chromophoric/fluorescent or easily transformed into pigmented or fluorescent compounds.

We believe that with the help of these combinatorial approaches plus effective high-throughput screening methods, researchers will be able to achieve even better results on improving microorganism performance under stress or enhancing biochemical yield.

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