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Review Article

# Harnessing microbial heterogeneity for improved biosynthesis fueled by synthetic biology

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ABSTRACT

Metabolic engineering-driven microbial cell factories have made great progress in the efficient bioproduction of biochemical and recombinant proteins. However, the low efficiency and robustness of microbial cell factories limit their industrial applications. Harnessing microbial heterogeneity contributes to solving this. In this review, the origins of microbial heterogeneity and its effects on biosynthesis are first summarized. Synthetic biology-driven tools and strategies that can be used to improve biosynthesis by increasing and reducing microbial heterogeneity are then systematically summarized. Next, novel single-cell technologies available for unraveling microbial heterogeneity and facilitating heterogeneity regulation are discussed. Furthermore, a combined workflow of increasing genetic heterogeneity in the strain-building step to help in screening highly productive strains - reducing heterogeneity in the production process to obtain highly robust strains (IHP-RHR) facilitated by single-cell technologies was proposed to obtain highly productive and robust strains by harnessing microbial heterogeneity. Finally, the prospects and future challenges are discussed.

## 1. Introduction

Constructing highly productive and robust strains is the foundation for efficient bioproduction of biochemical and recombinant proteins, which requires obtaining microorganism strains with a high titer, yield, and productivity, meanwhile maintaining the highly productive state stably during fermentation [1–3]. However, in the cultivation of a single strain, growth and metabolism heterogeneity can often be observed due to genetic variation induced by plasmid loss and mutation as well as non-genetic variation caused by stochastic gene expression, asymmetric cell division, copy number variation, and heterogeneous local environments [4,5]. In terms of biosynthesis of target products, microbial heterogeneity is a double-edged sword. It can be increased to generate libraries of diverse variants, from which highly productive strains can be obtained through growth-based directed evolution or fluorescence signal-based high-throughput screening [6–8]. It can also be increased to improve biosynthesis efficiency through metabolic division of labor [9,10]. Unfortunately, microbial heterogeneity can also be deleterious. During the fermentation of highly productive strains, microbial heterogeneity can cause the emergence of low- and non-productive cell subpopulations. Because biosynthesis of target biochemicals or recombinant protein often confers metabolic stress on engineered strains and impairs cell growth, emerging low- and non-productive cells have higher growth rates and can gradually replace high-productive cells during long-term or large-scale fermentation, therefore restricting bioproduction robustness and then hampering the industrialization of bioprocess [11,12]. Therefore, increasing microbial heterogeneity can facilitate the building of highly productive strains, and reducing heterogeneity can improve production robustness. Proper regulation of microbial heterogeneity is expected to obtain highly productive and robust strains.

Fueled by synthetic biology, efficient tools and strategies to increase and reduce microbial heterogeneity have recently been developed (Table 1). In addition, novel single-cell technologies for unraveling

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**Table 1**
Tools and strategies to harness microbial heterogeneity.

| Categories | Tools and strategies | Description | Refs |
|---|---|---|---|
| Increasing genetic heterogeneity | *In vivo* mutagenesis plasmid | A potent, inducible, broad-spectrum mutagenesis plasmid for *E. coli* constructed by manipulating DNA methylation state, cytosine deamination, base-excision repair and mutagenic nucleobase export. | [47] |
| | Genome replication engineering-assisted continuous evolution (GREACE) | A strategy to accelerate the evolution process in *E. coli* by introducing *dnaQ* (proofreading elements of the DNA polymerase complex) mutant library to disturb genome replication. | [49] |
| | A synthetic SIM module | A strategy to accelerate *E. coli* adaptive evolution by harnessing stress-induced mutagenesis (SIM). A genetic toggle switch was used to control the expression of the genes related to SIM in a bistable manner. | [48] |
| | Autonomous evolution mutation system (AEMS) | A system to promote the occurrence of mutations in *B. subtilis*. The system uses hierarchical dynamic control to switch between the high-fidelity module and the mutagenic module. | [7] |
| | Feedback-regulated evolution of phenotype (FREP) | An adaptive evolution system with autonomously adjustable mutation rates. Target product-responsive biosensor was used to control mutator gene *mutD5* so that the mutation rate increased in the absence of the target product to generate diversity in the population and reduced in the presence of the target product with a high concentration. | [50] |
| | Self-directed evolution based on a digital pH-sensing system | A directed evolution system with adjustable mutation rates depending on intracellular pH environments. | [6] |
| | CRISPR- and RNA-*assisted in vivo*-directed evolution (CRAIDE) | A CRISPR-assisted evolution system with RNA, not DNA, as a repair template. Variants of chimeric donor gRNAs composed of gRNA guiding Cag9/dCas9 and the RNA segment as a repair template, which are continuously transcribed by an error-prone T7 RNA polymerase, are used to introduce mutations by RNA-mediated repair. | [60] |
| | OrthoRep | A system with an orthogonal DNA polymerase–plasmid pair in yeast. It stably and orthogonally mutates at a rate about 100,000-fold faster than the host genome *in vivo*. | [61] |
| | Highly error-prone DNA polymerase I-based targeted gene evolution | A system resulting in a strong mutagenesis of a target sequence encoded in a Pol I-dependent plasmid. Point mutations that can increase the error rates of DNA polymerase I (Pol I) replication were introduced. | [62] |
| | Retrotransposon Ty1-based *in vivo* continuous evolution | A retrotransposon Ty1-based system that can be used for *in vivo* continuous evolution of genes and pathways in yeast. *In vivo* continuous evolution was enabled by coupling with growth selection. | [63] |
| | T7-targeted dCas9-limited *in vivo* mutagenesis (T7-DIVA) system | An *in vivo* evolution platform-based T7 RNA polymerase (T7RNAP) and catalytically dead Cas9 (dCas9) in *E. coli*. The platform uses T7 RNA polymerase to target mutagenic enzymes (base deaminase) to the target sequence and uses catalytically dead Cas9 (dCas9) combined with custom-designed crRNAs as a "roadblock" to constrict the size of the mutation window. | [64] |
| | Expanded MutaT7 toolkit | Targeted mutagenesis platforms mediated by nucleotide base deaminase-T7 RNA polymerase fusions with higher mutation frequencies and expanded utility. | [66] |
| | Targeted *in vivo* diversification enabled by T7 RNAP (TRIDENT) | A mutagenesis platform that uses T7 RNA polymerase to target two different types of base deaminases to the target sequence to broaden the mutation spectra and localize DNA repair factors to sites of deaminase-driven mutations to enhance the mutation rate. | [65] |
| Increasing non-genetic heterogeneity | Asymmetry distribution-based synthetic consortium (ADSC) | An asymmetry distribution-based synthetic consortium (ADSC) that can coordinate the ratio of production cells and growing cells in the population by asymmetric cell division, thereby improving production by metabolic division of labor. | [10] |
| | Integrase-mediated differentiation circuits | Circuits that divide the population into two cell types, progenitors (responsible for replication and proliferation) and | [9] |

(*continued on next page*)

**Table 1** (*continued*)

| Categories | Tools and strategies | Description | Refs |
|---|---|---|---|
| | | differentiators (responsible for biosynthesis) by terminal differentiation, therefore improving the evolutionary stability of burdensome and toxic functions in *E. coli*. | |
| | Asymmetric plasmid partitioning-based asymmetric cell division | A synthetic system for asymmetric cell division in *E. coli* based on asymmetric plasmid partitioning from *C. crescentus*. | [72] |
| Reducing genetic and non-genetic heterogeneity | Synthetic symbiosis combining plasmid displacement | A strategy to construct a phenotype-stable microbial system. It maintains plasmid stably by expressing essential genes and genes of interest in the same plasmid backbone and uses plasmid displacement to simplify the workflow. | [74] |
| | Choosing appropriate host cells | Different host cells have different characteristics, and choosing appropriate host cells is also a promising way of reducing microbial heterogeneity. | [76,77] |
| | Constructing chassis with reduced mutation rates | Deleting or inhibiting unstable elements in the genome, including prophage, insertion sequence elements and error-prone DNA polymerases, is a promising approach to constructing chassis with reduced mutation rates. | [83–87] |
| | Deleting *recA* | Deleting *recA helps* construct robust chassis with reduced homologous recombination rate to express multicopy genes stably. | [89] |
| | Periodic reselection for evolutionarily reliable variants (PResERV) | A directed evolution strategy for characterizing new targets that replicate ColE1-type plasmids with higher fidelity in *E coli* | [90] |
| | Combinatorial assembly platform | A strategy to overcome genetic instability, which requires the construction of a library of metabolic pathway-encoding variants using efficient DNA assembly methods. Then, stable variants were selected from the library. | [78] |
| | Metabolic coupling of cell growth with biosynthesis | A pyruvate-driven growth-coupled strategy to improve bioproduction robustness. By deleting endogenous pyruvate-releasing pathways, *E. coli* was engineered to use the target product biosynthesis pathway as the sole endogenous pyruvate-releasing pathway to support growth. | [79] |
| | Synthetic sequence entanglement | A strategy to constrain the evolution path by overlapping a sequence of interest with an essential gene. | [80] |
| | Synthetic addiction based on product-responsive biosensor | Strategies to couple cell growth with biosynthesis by using the (intermediate or end) product-responsive biosensor to control the expression of growth-related key genes, such as essential genes and amino acid synthesis-related genes. | [12,40, 81,82] |
| | Engineering global gene regulatory | A strategy to enable homogeneous expression of green fluorescent protein (GFP) in *Bacillus subtilis* by $CodY^{R214C}$ mutation | [91] |
| | Engineered promoters with constant gene expression at any copy number in bacteria | Gene parts overcoming heterogeneity caused by copy number variation between individual cells. An incoherent feedforward loop (iFFL) was engineered into *E. coli* promoters using transcription-activator-like effectors (TALEs). | [93] |
| | Stable and tunable plasmid (STAPL) system | A system used to minimize cell-to-cell variation of a plasmid-based expression system under antibiotic-free conditions. The essential gene *infA* is encoded on the plasmid instead of the chromosome. | [20] |
| | (Inducible) Population quality control | Strategies to exploit non-genetic heterogeneity to improve biosynthesis. Coupling of growth with biosynthesis was used to enrich high-performing cell subpopulations. | [11,40] |

microbial heterogeneity are available and widely used. These advances facilitate microbial heterogeneity analysis and regulation. As for microbial heterogeneity occurring in the cultivation of a single strain, previous reviews have mainly focused on the origins and functional roles of microbial heterogeneity, tools for the measurement and analysis of microbial heterogeneity and just one side of harnessing genetic heterogeneity (increasing genetic heterogeneity to construct diverse libraries of mutants or reducing genetic heterogeneity for highly robust strains) [4,13–17]. However, the synthetic biology-driven tools and strategies that can be used to improve biosynthesis by increasing and reducing genetic and non-genetic heterogeneity have not been systematically summarized and discussed, especially how to cooperatively utilize two sides of harnessing heterogeneity (increasing and reducing) for highly productive and robust strains, and how to use novel single-cell technologies to facilitate microbial heterogeneity regulation. Therefore, a discussion on current progress and future perspectives of increasing and reducing microbial heterogeneity for improved biosynthesis fueled by synthetic biology is now relevant.

By exploiting the advantages of different species, synthetic microbial consortia based on co-cultivation of multiple species also use heterogeneity to achieve sophisticated functions for reduced metabolic burden [18] and high substrate conversion capacity [19]. Although gaining much attention, co-cultural microbial consortia are very different from

microbial heterogeneity occurring in the cultivation of a single strain. Here, we only focus on the latter. This review summarizes the origin of microbial heterogeneity and its impact on biosynthesis. It highlights advances in synthetic biology-driven tools and strategies and novel single-cell technologies that aid in understanding and regulating microbial heterogeneity. Furthermore, a combined workflow facilitated by single-cell technologies, namely increasing genetic heterogeneity in the step of strains building to help in building highly productive strains - reducing heterogeneity in the production process to obtain highly robust strains (IHP-RHR) was proposed to obtain highly productive and robust strains by harnessing microbial heterogeneity. Finally, prospects and future challenges were proposed.

## 2. The origins of microbial heterogeneity and its effects on biosynthesis

### 2.1. Genetic heterogeneity

Constructing microbial cell factories to synthesize chemicals based on plasmid expression systems and genome engineering has enabled the renewable production of various valuable products, such as pharmaceuticals, nutraceuticals and biofuel, among others [1–3]. However, in plasmid-based strains, plasmid loss generates non-productive cells, resulting in a heterogeneous production population in the fermentation [20]. In addition, even if the plasmid can be maintained stably or genome engineering has enabled microorganism-based bio-production, mutagenesis, an intrinsic feature of microorganisms, can also result in a heterogeneous population. In the growth phase of microorganisms, a variety of mutations can occur due to intrinsic DNA polymerase errors, insertion sequence (IS) elements, DNA deamination and the activities of error-prone DNA polymerases, among others (Fig. 1) [21–23]. Furthermore, the mutation rate can vary depending on the environment. Spontaneous mutation rates have been reported to be negatively associated with population density in both eukaryotes and bacteria, with mutation rates up to 23-fold lower at high population densities, a phenomenon called density-associated mutation-rate plasticity (DAMP) [24]. Furthermore, adaptive mutation (also known as stress-associated mutation) has been found to exist widely in microorganisms [25,26]. It is a phenomenon that microbial mutation rates tend to increase when they are in stressed environments, such as antibiotic treatment, nutrition starvation and metabolic stress, among others [25–29]. As a result of plasmid loss and mutagenesis, a microbial population can exhibit genetic heterogeneity. Genetic heterogeneity caused by mutagenesis can generate strains with diverse phenotypes, which provides a library for screening highly productive strains. Adversely, plasmid loss and mutations occurring in the scale-up of high-productive strains always generate low- and non-productive cells, which cell cultivations tend to select for [12,30]. Therefore, genetic heterogeneity can also hamper biosynthesis robustness.

### 2.2. Non-genetic heterogeneity

The other microbial heterogeneity is non-genetic (also called phenotypic heterogeneity). With the advancement of single-cell

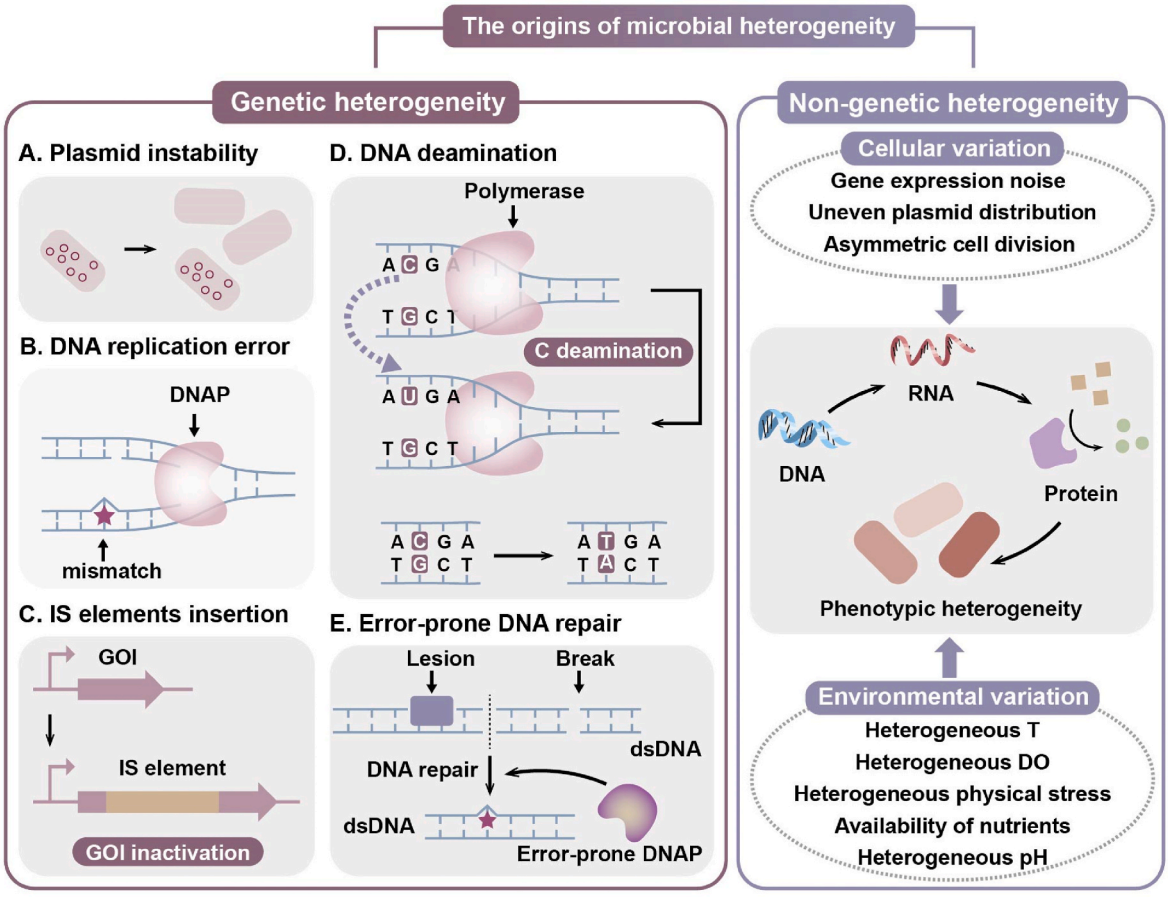


**Fig. 1.** The origins of microbial heterogeneity. Microbial heterogeneity includes genetic and non-genetic heterogeneity. Genetic heterogeneity can result from plasmid instability, DNA replication error, gene of interest (GOI) inactivation by IS element, DNA deamination of single-strand DNA (ssDNA), and error-prone DNA polymerase (DNAP)-mediated DNA repair. Cellular and environmental variations can both result in non-genetic heterogeneity by influencing gene expression. The cellular variations include gene expression noise, uneven plasmid distribution, and asymmetric cell division. The environmental variations include heterogeneous temperature (T), dissolved oxygen (DO), pH and physical stress, as well as heterogeneity in the availability of nutrients.

technologies, such as flow cytometry and single-cell time-lapse microscopy, it has been accepted that the isogenic cell population exhibits tremendous phenotypic heterogeneity to tackle rapidly changing environments [31–33]. Although it often occurs when the local microenvironment varies [4], non-genetic heterogeneity also exists when isogeneic cells are under identical conditions due to gene expression noise [34,35], asymmetric cell division [36,37], and heterogeneous plasmid distributions (Fig. 1) [38,39]. On the one hand, non-genetic heterogeneity can be increased to improve biosynthesis efficiency by metabolic division of labor [9,10]. On the other hand, non-genetic heterogeneity in biosynthesis performance can occur in the cultivation of production strains, which results in the co-existing of high-, low- and non-productive cell subpopulations in the culture, therefore restricting the robustness of bioprocess [11,40]. Although low- and non-productive cell subpopulations caused by non-genetic variation have been observed to resume production at an unpredictable time [41], cell cultivation tending to select against high-productive cells with metabolic burden and reduced fitness exacerbates the adverse effects of non-genetic heterogeneity [11,40].

Therefore, increasing microbial heterogeneity in the step of strain building can help to obtain highly productive strains and reducing microbial heterogeneity in the production process is promising to improve bioproduction robustness. Furthermore, synthetic biology has fueled the development of efficient tools and strategies for increasing and reducing microbial heterogeneity. Therefore, synthetic biology-driven tools and strategies that can be used to improve biosynthesis by increasing and reducing microbial heterogeneity are then systematically summarized in the following sections.

## 3. Tools and strategies to increase microbial heterogeneity

### 3.1. Increasing genetic heterogeneity to construct diverse libraries of mutants

Most metabolic engineering strategies are rationally designed based on the identified gene targets for engineering. However, identification of gene targets can be difficult due to the complexity and interconnectivity of metabolic networks [42]. Therefore, such rational design strategies are time-consuming and not always effective [43]. Directed evolution and high-throughput screening avoid the aforementioned problems by screening highly productive strains from diverse libraries of mutants based on cell growth and fluorescence signals [44,45]. With the development of synthetic biology, many strategies and tools have been developed to increase microbial genetic heterogeneity via mutation regulation to efficiently or accurately generate variant libraries, which is the prerequisite of directed evolution and high-throughput screening [46]. According to mutation sites, those tools and strategies mainly involve two categories, increasing heterogeneity in an untargeted way and increasing heterogeneity in a targeted way.

One of the most commonly used methods of increasing genetic heterogeneity in an untargeted way is to construct a mutator strain with enhanced basal mutation rates [6,7,47,48]. Mutator strains can be obtained by deletion or repression of genes involved in DNA replication and repair, perturbation of deoxyribonucleoside triphosphate (dNTP) pool and overexpression of error-prone DNA polymerase. By manipulating DNA methylation state and cytosine deamidation, reducing base-excision repair and compromising intracellular dNTP pools etc., Badran et al. [47] developed a potent *in vivo* mutagenesis system with a broad mutagenesis spectrum, a very high mutation rate and a wide application in *E. coli*. It enabled the evolution of antibiotic resistance in wild-type *E. coli* within 24 h and successfully evolved T7 RNA polymerase to initiate transcription of T3 promoter within 10 h.

However, a high basal mutation rate also brings numerous drawbacks, including poor transformation efficiency, slow growth rate and impaired strain stability, due to the accumulation of disadvantageous and deleterious mutations [47]. Therefore, some strategies that enable switching between high and low basal mutation rates have been developed. Based on a mutator plasmid carrying error-prone mutant of *dnaQ* encoding ε subunit of *E. coli* DNA polymerase III, genome replication engineering assisted continuous evolution (GREACE) has been developed to improve *n*-butanol and acetate tolerances of *E. coli*, which switches from high mutation rate to low mutation rate by curing of the mutator plasmid [49]. In addition, changing mutation rates have been achieved in *E. coli* and *Bacillus subtilis* by inducible promoters. Mutator genes and fidelity genes were under the control of different inducible promoters (Fig. 2A). By inducing the expression of mutator genes into a high mutation rate state or expression of fidelity genes into a low mutation rate state, those systems improved *n*-butanol production in *E. coli* and acetoin production in *B. subtilis* by periodic adaptive evolution [7,47,48].

To develop autonomous mutagenesis systems, product-responsive biosensors have been used to regulate the expression of mutator genes to adaptively tune mutation rates based on product titer [50]. Specifically, when the product titer is low, the mutator gene is actively expressed to increase genetic heterogeneity in the population. Contrarily, the expression of the mutator gene is inhibited at a high product titer to enable stable inheritance of a high-performing phenotype (Fig. 2B). It has been reported that acid-tolerant microorganisms adapt to extreme extracellular pH by maintaining a near-neutral cytosolic environment [51,52]. Therefore, a similar system using a pH-responsive riboswitch, which tunes the mutation rate according to the intracellular pH level, has been developed to improve the acid tolerance of *E. coli* [6]. In this circuit, an error-prone mutant of dnaQ is actively expressed in the acidic intracellular pH environment to increase genetic heterogeneity. The expression of an integrase allows the *dnaQ* mutant cassette to be flipped to an inactive conformation in the near-neutral cytosolic environments of acid-tolerant mutants emerging in the heterogeneous population, which enables stable inheritance of acid-tolerant phenotype.

The aforementioned strategies can achieve unbiased mutation that affects the entire genome, therefore, they are susceptible to “cheater” mutations. The “cheater” mutations do not confer the desired phenotype on the host cells but allow them to escape from selection and screening [46]. Therefore, many strategies have been developed to increase genetic heterogeneity in a targeted way, based on the combination of CRISPR systems and oligo pools, single-base editors, orthogonal DNA replication systems (Fig. 2D) and targeted retrotransposon elements (Fig. 2E). These tools and strategies have been discussed in detail elsewhere [46,53]. Recently, novel genetic mutation tools based on RNA-mediating DNA repair and T7 RNA polymerase have been developed to evolve target proteins. Therefore, those novel tools are the main focus here.

Many CRISPR system-based mutagenesis technologies require *in vitro* designed and synthesized variant DNA donors [54–56]. Interestingly, in addition to DNA donors, RNA has been proven to be capable of mediating DNA double-strand break (DSB) repair as direct donors in bacteria and yeast [57–59]. Therefore, CRISPR- and RNA-assisted *in vivo* directed evolution (CRAIDE) has been developed for yeast [60]. Variants of chimeric donor gRNAs composed of gRNA guiding Cag9/dCas9 and the RNA segment as a repair template (temp-cgRNA) were continuously transcribed by an error-prone T7 RNA polymerase and then used to introduce mutations *via* RNA-mediated repair (Fig. 2C). It has been reported that orthogonal DNA replication systems and targeted retrotransposon element-based mutagenesis strategies have large mutation windows [61–63]; however, using them to mutate gene segments or domains poses a significant challenge. Although some CRISPR-based base editors can address the problem, their processivity is limited by their small mutation windows and therefore they are still limited for long gene segments or domains [64]. To address the problem, an *in vivo* evolution platform based on T7 RNA polymerase (T7RNAP) and catalytically dead Cas9 (dCas9) was developed in *E. coli* (Fig. 2F). T7 RNA polymerase is used to direct mutagenic enzymes (base deaminase, BD) to

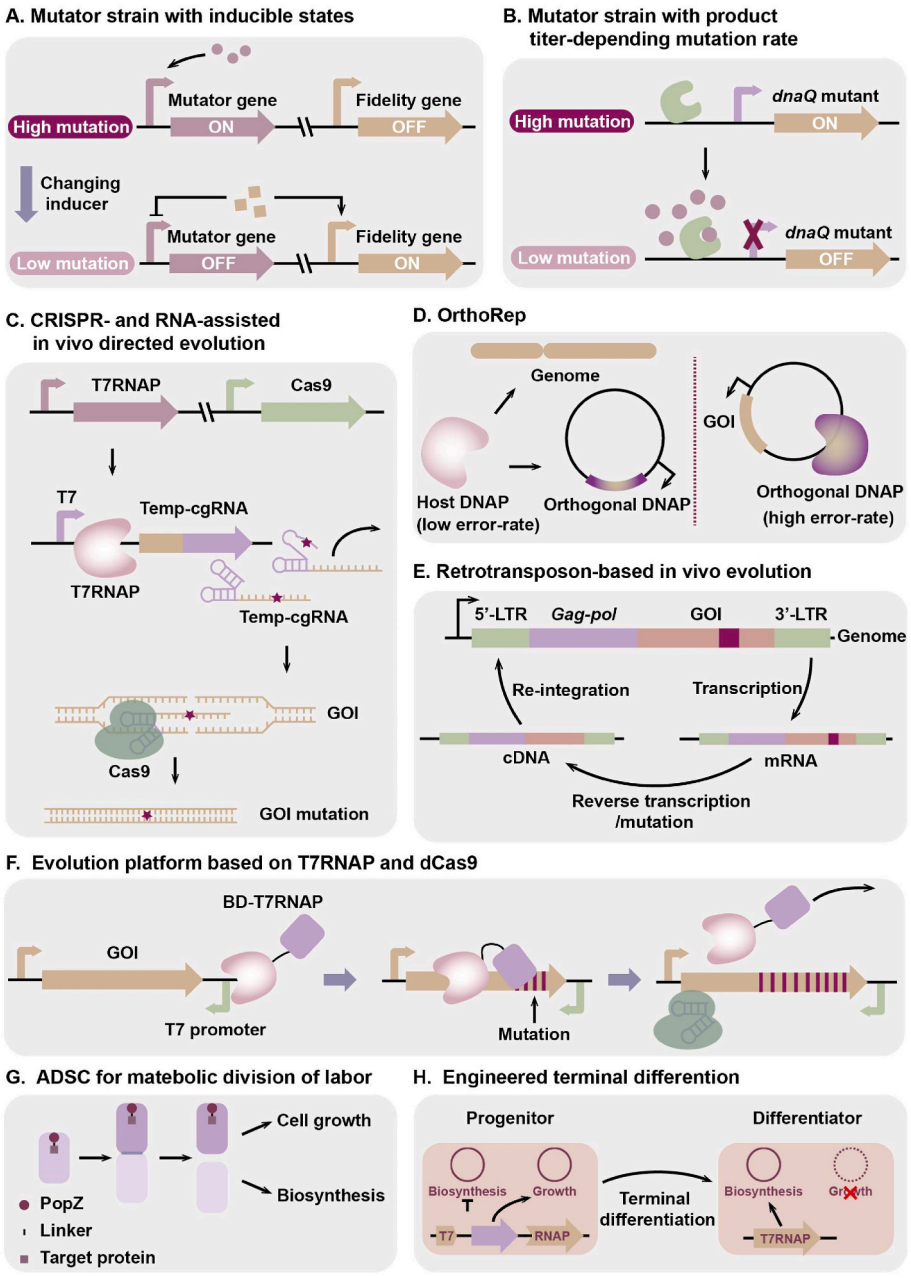


**Fig. 2.** Strategies and tools to increase microbial heterogeneity. A) Mutator strain with mutator gene and fidelity gene controlled by an orthogonal inducible promoter pair. One inducer is added to activate the mutator gene to increase the mutation rate to generate a mutant library. The other inducer can activate the fidelity gene and repress the mutator gene simultaneously to reduce the mutation rate to stably maintain the improved phenotype at the end of evolution. B) Mutator strain with product titer-dependent mutation rate. Product-responsive biosensors have been used to regulate the expression of the mutator gene to adaptively tune mutation rates based on the product titer. In the absence of the target product, the mutator gene is activated to increase the mutation rate to generate diversity in the population and is repressed to reduce the mutation rate when the titer of the target product is high. C) A CRISPR-assisted evolution system with RNA, not DNA, as a repair template. Variants of chimeric donor gRNAs composed of gRNA guiding Cag9/dCas9 and the RNA segment as repair template(temp-cgRNA), which are continuously transcribed by an error-prone T7 RNA polymerase, are used to introduce mutations by RNA-mediated repair. D) A system with an orthogonal DNA polymerase–plasmid pair in yeast. Host DNA polymerase (DNAP) replicates genome and orthogonal DNAP-carrying plasmid stably. Orthogonal DNAP replicates gene of interest (GOI)-carrying plasmid with a high mutation rate. E) A retrotransposon Ty1-based *in vivo* continuous evolution system. After the transcription of retroelement, the transcript including GOI is converted into cDNA in an error-prone manner. Then, the generated cDNA is re-integrated into the genomic locus, thereby introducing mutations. F) An *in vivo* evolution platform based on T7RNAP and catalytically dCas9. The platform uses T7RNAP to target mutagenic enzymes (base deaminase, BD) to the target sequence to introduce mutations and uses dCas9 combined with custom-designed crRNAs as a "roadblock" to constrict the size of the mutation window. G) An asymmetry distribution-based synthetic consortium (ADSC) that can coordinate the ratio of production cells and growing cells in the population by asymmetric cell division, thereby improving production by metabolic division of labor. H)A synthetic circuits that divide the population into two cell types, progenitors (responsible for replication and proliferation) and differentiators (responsible for biosynthesis) by terminal differentiation, therefore improving the evolutionary stability of burdensome and toxic functions in *E. coli*.

the target sequence. Meanwhile, crRNA-guided dCas9 can prevent transcriptional elongation by T7RNAP, preventing mutagenesis in the downstream region of the targeted sequence [64]. Furthermore, by using two different types of base deaminase, using their highly active mutants or localizing DNA repair factors to the mutation sites of deaminase-driven mutations, similar systems with higher mutation rates and broader mutation spectra were developed in yeast and *E. coli* [65,66].

### 3.2. Increasing non-genetic heterogeneity for metabolic division of labor

In addition to genetic heterogeneity, non-genetic heterogeneity can be increased to improve biosynthesis efficiency by metabolic division of labor. One of the methods of increasing non-genetic heterogeneity is to construct asymmetric cell division. It has been found that PopZ protein from *Caulobacter crescentus* often accumulated at a single pole of *E. coli* cells [67,68]. By fusing protein of interest to PopZ, *E. coli* cells can be differentiated into cells with different functions, achieving metabolic division of labor [69–71]. Based on that, an asymmetry distribution-based synthetic consortium (ADSC) was developed to improve shikimate synthesis (Fig. 2G) [10]. Shikimate can be accumulated by inactivating shikimate kinases (such as AroK), which catalyzes shikimate into the chorismate pathway to support cell growth. However, inactivating shikimate kinases can impair cell growth. To address the problem, ADSC was constructed by using a promoter with a TetO operator and a stationary phase promoter (SPP) to control the expression of PopZ-AroK fusion and TetR repressor, respectively. Before the stationary phase of cell growth, PopZ-AroK fusion was overexpressed due to the lack of TetR and cells were differentiated into two types, growing cells with PopZ-AroK fusion and productive cells without the fusion. In the stationary phase of cell growth, the expression of PopZ-Arok was repressed by transcriptional repressor TetR controlled by SPP. In this way, ADSC can coordinate the ratio of productive cells and growing cells in the population by using SPPs with different intensities, thereby improving shikimate production by metabolic division of labor. In addition to PopZ, Molinari et al. [72] developed a synthetic system for asymmetric cell division in *E. coli* based on asymmetric plasmid partitioning. In this system, the centromere-binding *trans*-acting protein (ParB) of *C. crescentus* binds to a centromere-like *cis*-acting sequence (parS) and gathers together plasmids with parS to form a partition complex. Upon cell division, only one of the daughter cells keeps the partition complex and the other daughter cell loses the plasmid with parS, generating two cell types. In this way, the physical separation of genetically distinct cells was achieved by tying motility to differentiation. However, the merits of asymmetric plasmid partitioning-based asymmetric cell division on biosynthesis still need to be verified experimentally.

Furthermore, metabolic division of labor can also be achieved by terminal differentiation. Engineered terminal differentiation in a single strain can facilitate the production of functional toxic proteins by improving their evolutionary stability [9]. Productions of functional toxic proteins often face great challenges. Because populations expressing functional toxic protein are susceptible to loss of function due to mutagenesis and selections for non-productive mutants with growth advantages. Recognizing that the production of functional toxic protein and cell proliferation must occur in the same cells to select for non-productive mutants, Williams et al. [9] developed integrase-mediated differentiation circuits (Fig. 2H) to divide an *E. coli* population into two cell types, progenitors and differentiators by terminal differentiation. Progenitors were responsible for replication, proliferation and incapable of production due to the absence of functional T7 RNAP, and differentiators were responsible for expressing functional toxic protein. Bxb1 integrase was used to execute the differentiation function by recombination in progenitors. Upon differentiation, functional T7 RNAP was formed to initiate transcription of the functional toxic protein. Meanwhile, *pir* cassette encoding π-protein would be excised and R6K plasmid relying on π-protein and conferring chloramphenicol resistance on cells would lose replication ability. In the presence of chloramphenicol selection, generated differentiators with production ability only have limited growth due to the loss of plasmid R6K plasmid, therefore non-productive mutants emerging from the differentiators cannot expand in the population. By tuning the expression of Bxb1 integrase through changing inducer concentration and concentration of chloramphenicol in the medium, production rate and duration of production were able to be coordinated to achieve optimal production of functional toxic protein. Although the genotype of differentiators is different from progenitors due to the recombination mediated by Bxb1 integrase and loss of R6K plasmid, genotypes of the differentiators are unable to inherit stably as their limited growth in the presence of chloramphenicol selection. Therefore, we classify the integrase-mediated differentiation circuits as the tools to increase non-genetic heterogeneity.

## 4. Tools and strategies to reduce microbial heterogeneity

Microbial heterogeneity in biosynthesis can manifest as the emergence of non- or low-productive cell subpopulations in the fermentation of high-productive strains, thereby impairing the robustness of biosynthesis. Therefore, it is promising to improve production robustness by reducing microbial heterogeneity in the production process. Next, we discuss the tools and strategies that can be used to improve bioproduction robustness by reducing microbial heterogeneity, including reducing genetic heterogeneity by addressing plasmid instability and mutagenesis as well as reducing non-genetic variation by homogeneous gene expression systems and genetic circuits (Fig. 3).

### 4.1. Reducing genetic heterogeneity caused by plasmid instability

Although susceptible to plasmid instability, plasmid-based systems are still widely used due to the convenience and high efficiency of manipulations. Traditionally, plasmids are maintained stably by expressing resistance genes to support cell growth in the antibiotic-supplemented medium. Antibiotic-free systems, such as auxotrophic complementation, post-segregational killing (PSK), operator–repressor titration (ORT) and RNA-based interactions, among others, have been developed because antibiotic use is environmentally unfriendly and costly [73]. However, those systems need to engineer host metabolism and tune the dosage and interaction of parts finely, lacking portability and efficiency. To address the problems, a strategy with convenience and broad applications called synthetic symbiosis combining plasmid displacement has been developed [74]. It keeps the plasmid stable by expressing essential genes and genes of interest in the same plasmid backbone. Specifically, the system first created a temperature-sensitive plasmid expressing an essential gene, which was then transformed into *E. coli*. Subsequently, a platform strain was created by removing the essential gene copy from the genome. The production plasmid carrying both the essential gene and pathway genes was then introduced into the platform strain. Finally, the temperature-sensitive plasmid was cured by adjusting the incubating temperature, and the essential gene in the production plasmid took over to support cell growth. Taking salicylic acid production as the case study, the system assisted in maintaining a high-performing phenotype for up to 80 generations.

### 4.2. Reducing genetic heterogeneity caused by mutagenesis

The most direct approach to reducing microbial heterogeneity caused by mutagenesis is to choose DNA sequences without short repeats and mononucleotide stretches as well as to avoid genetic part reuse [75]. Choosing the appropriate host cells is also a promising approach. Taking mevalonate and 2,3-butanediol production as the case study, short- and long-read ultra-deep sequencing was performed to profile emerging heterogeneity in five platform *E. coli* strains [76]. It has been

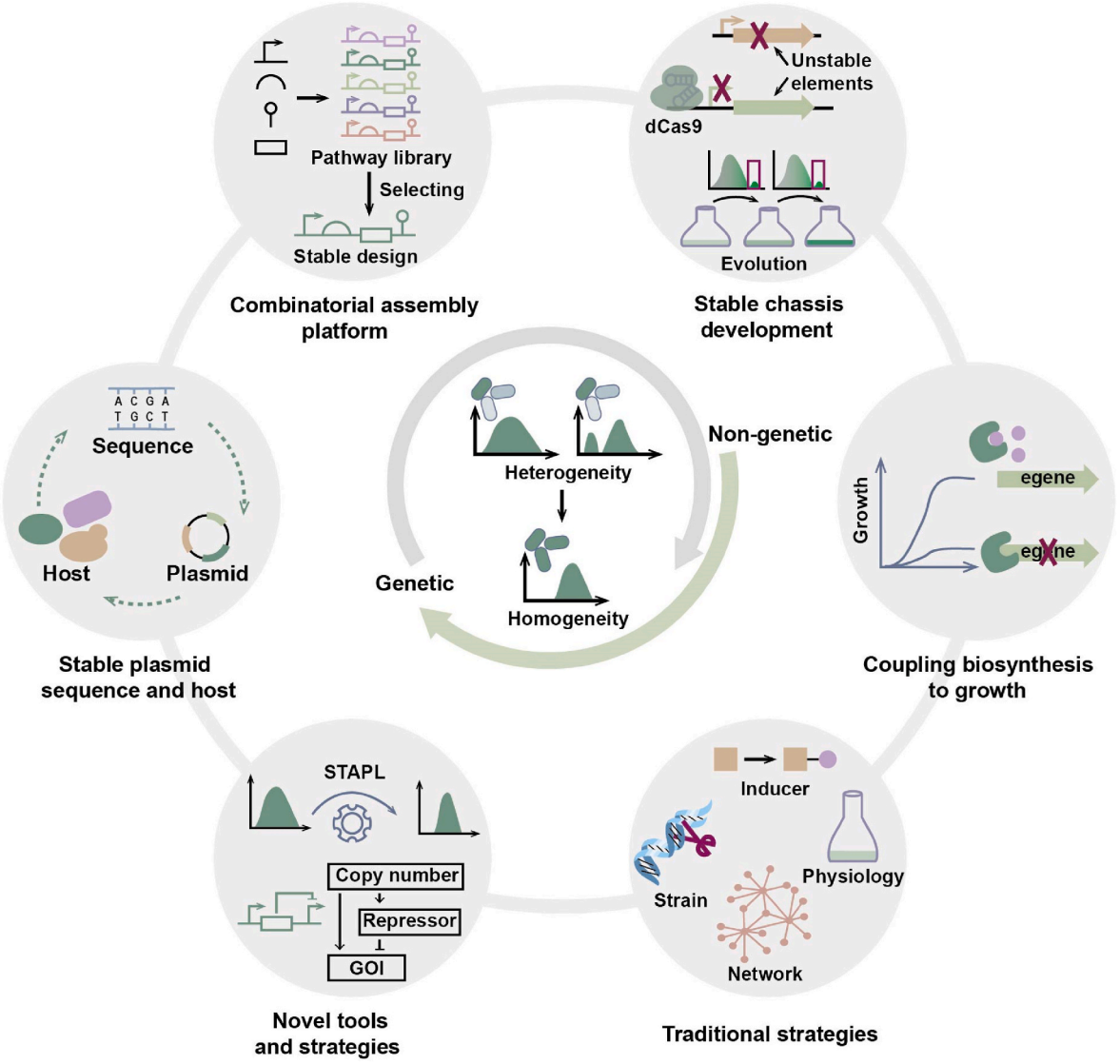


**Fig. 3.** Strategies to reduce microbial heterogeneity. The widely used methods of reducing microbial genetic heterogeneity include maintaining the stability of plasmid, choosing stable DNA sequence and host, using a combinatorial assembly platform to construct and select a stable pathway design, coupling biosynthesis with growth and developing stable chassis with reduced mutation rate. The strategies to address non-genetic heterogeneity include the traditional strategies, such as engineering global gene regulatory, strain and inducer modifications and physiology manipulations, and novel tools and strategies for maintaining homogeneous gene expression. In addition, coupling growth with biosynthesis can also be an efficient and effective strategy to address non-genetic heterogeneity.

suggested that different chassis strains exhibit varying product characteristics and robustness for the same metabolic pathway, and chassis strains with active IS elements, such as *E. coli* TOP10, are unsuitable for bioproduction [76]. It has been reported that when expressing the ethylene-forming gene *efe* from *Pseudomonas syringae*, *S. elongatus* PCC 7942 population exhibited genetic heterogeneity, including two cell subpopulation - one synthesizing ethylene and one containing a truncated *efe* gene and not synthesizing ethylene. However, using *Synechocystis* sp. PCC 6803 to express the *efe* prevented the genetic instability observed in *S. elongatus* PCC 7942 [77], also proving the importance of choosing the appropriate host cells. Unbalanced expression of pathway enzymes has been known to impair cell growth by accumulation of toxic intermediates or depletion of precursor metabolites for growth. Therefore, unbalanced metabolic pathways are genetically unstable. For this phenomenon, using libraries of synthetic promoters, ribosomal binding sites and terminators, Taylor et al. [78] employed a combinatorial assembly platform to construct a library of lycopene pathway-encoding variants of cyanobacteria. Then, stable variants were selected from the library. After a single combinatorial round, 80 % of the variants randomly chosen maintained the bioproduction phenotype over many generations.

Coupling cell growth with biosynthesis is also a promising strategy for improving bioproduction robustness. The coupling confers more growth advantages on producing cells and improves production robustness by enriching productive cells during fermentation. For example, a pyruvate-driven metabolic scenario for coupling cell growth with biosynthesis has been achieved in *E. coli* to boost the robust production of anthranilate and its derivatives. By deleting endogenous pyruvate-releasing pathways, *E. coli* was engineered to couple cell growth with anthranilate biosynthesis by using the anthranilate biosynthesis pathway as the sole endogenous pyruvate-releasing pathway to support growth [79]. However, due to the complexity and unpredictability of the metabolic network, developing a metabolic scenario for coupling cell growth with biosynthesis still faces a great challenge. Therefore, engineering growth-related cell processes to couple growth with biosynthesis has become more prevalent. For example, co-expressing pathway genes and an essential gene in a polycistron form or overlapping a key pathway-encoding gene with an

essential gene both achieved enhanced genetic stability of *E. coli* [30,80]. With the development of synthetic biology, an increasing number of metabolite-responsive biosensors have been developed and optimized. One of the most commonly used is transcription factor (TF)-based biosensors that are composed of TF and the sensor promoter with binding sites of TF. The former can regulate transcription of the latter by responding to metabolites specifically. Population quality control system (PopQC) and synthetic addiction have been developed for *E. coli*, *B. subtilis* and yeast, which couple growth with biosynthesis by using TF-based biosensors responding intermediate or end products to control the expression of growth-related key genes, such as essential genes and amino acid synthesis-related genes [12,40,81,82]. In the two systems, highly productive cells have more growth advantage due to the expressions of growth-related key genes activated by intracellular (intermediate or end) products compared to non- and low-productive mutants, in which the expressions of growth-related key genes are inhibited. This way, genetic heterogeneity in biosynthesis has been overcome by enriching highly productive cells.

Although it is an effective and efficient way to improve production robustness, growth coupling with biosynthesis is susceptible to mutation. To address this problem, Cao et al. [40] developed double-output inducible population quality control (iPopQC). In PopQC, the transcription factor responding target product was controlled by a constitutive promoter. Therefore, once cell growth and biosynthesis are coupled successfully, the target product needs to be added to the medium to support cell growth in the non-producing phase, which makes PopQC costly and only suitable for products capable of entering into the cytoplasm [12,40]. To expand the applicability, iPopQC uses an inducible promoter to control the expression of the transcription factor responding target product, and it only functioned with inducer addition. In addition, by using the sensor promoter to control two essential genes in two expression cassettes, double-output iPopQC co-coupled the expression of double essential genes with biosynthesis, which exhibits better robustness than single-output iPopQC coupling the expression of one essential gene with biosynthesis. In addition, chassis with a reduced mutation rate may be another promising method of reducing microbial genetic heterogeneity. It has been reported that deleting or inhibiting unstable elements in the genome, including prophage, IS elements, and error-prone DNA polymerases, is a widely used approach to reducing mutation rate [83–87]. However, no experiments have yet shown that these strategies are capable of maintaining high-productive phenotypes over many generations. In addition, by knocking out or inhibiting *recA*, a robust chassis with a reduced homologous recombination rate was developed to express multicopy genes stably [88,89]. However, it is still difficult to construct a chassis with a very low mutation rate using rational strategies due to the complexity of DNA replication and repair mechanisms. A directed evolution strategy called periodic reselection for evolutionarily reliable variants (PResERV) was developed to characterize new targets that replicate ColE1-type plasmids with higher fidelity in *E. coli* to solve this problem [90]. In PResERV, *E. coli* expressing costly fluorescent protein was first mutagenized and then the mutants remaining fully fluorescent over tens to hundreds of cell divisions were selected periodically by flow cytometry-based fluorescence-activated cell sorting. Next, genome sequencing was performed. Some mutations in DNA polymerase I and in RNase E that reduce the mutation rates of ColE1-type plasmids were characterized, providing new insights into improving the genetic stability of ColE1-type plasmids in *E. coli*.

### 4.3. Reducing non-genetic heterogeneity

In addition to genetic heterogeneity caused by plasmid loss and mutagenesis, non-genetic heterogeneity often exists in isogeneic populations. Engineering global gene regulatory networks is a promising solution for the phenotypic variation of heterologous protein expression. When expressing green fluorescent protein (GFP) under the control of isopropyl-β-D-thiogalactosidase (IPTG) -induced promoter, wild-type *B. subtilis* has been reported to be prominently heterogeneous during the late stationary phase. This heterogeneity is correlated with the pleiotropic transcriptional regulator CodY, which regulates genes involved in nitrogen metabolism by specifically binding to its operator located in the promoter region of target genes. The introduction of mutation R214C in CodY resulted in the uniform expression of GFP between individual cells by reprogramming the metabolic regulatory networks to reduce the metabolic burden of protein expression [91]. Traditional strategies addressing non-genetic heterogeneity also include choosing a stable plasmid to avoid uneven plasmid distribution, strain modifications, inducer modification and physiology manipulation (such as adjusting cultivation mode, medium and inducer concentration et al.), among others, which have been discussed in detail elsewhere [92]. Therefore, the state-of-the-art tools and strategies for overcoming non-genetic heterogeneity, including promoters with constant gene expression at any copy number, as well as a stable and tunable plasmid (STAPL) system et al., were mainly reviewed next.

For plasmid-based systems, gene copy number heterogeneity is a common source of non-genetic variation. To ensure homogeneous expression, an incoherent feedforward loop (iFFL) was engineered into *E. coli* promoters using transcription-activator-like effectors (TALEs). Specifically, TALE proteins can be designed to bind 18-bp operators and repress transcription of the promoter with the operator. Engineered promoters were constructed by placing the operator in a strong constitutive promoter with varied locations and orientations and recoding TALE upstream of the strong promoter. Copy number variation influences the expression of the TALE and then the latter counteracts the effect of copy number variation on the expression of the engineered promoters as a repressor, which forms an iFFL. In this way, these promoters achieved a homogeneous expression at any copy number. When directly moved from a plasmid into the genome, the deoxychromoviridans pathway genes regulated by the engineered promoters retained similar function, immune to copy number variations [93]. In addition, Kang et al. developed a stable and tunable plasmid (STAPL) system for *E. coli*, which expresses the essential gene *infA* on a plasmid instead of the chromosome to stably maintain the plasmid copy number under antibiotic-free conditions. Moreover, varying the expression level of *infA* enabled rational tuning of plasmid copy number. Compared to cultivated with antibiotics, STAPL resulted in a 2-fold increase in the production of itaconic acid and lycopene under antibiotic-free conditions [20]. Furthermore, for non-genetic heterogeneity in biosynthetic performance, coupling growth with biosynthesis based on product-responsive biosensors can also be efficient and effective, as illustrated in population quality control (PopQC) [11,40].

## 5. Emerging single-cell technologies for facilitating harnessing microbial heterogeneity

Novel single-cell analytical technologies that can be employed to explore microbial heterogeneity are becoming more available and widely used. They can be divided into two categories, single-cell phenotypic analysis technologies such as single-cell technologies analyzing DNA replication-related mutation rate and biosynthesis, and single-cell transcriptome sequencing technologies. These single-cell technologies can be employed to monitor microbial heterogeneity, screen highly productive phenotypes and analyze the mechanism of microbial heterogeneity, which can in turn provide novel targets for harnessing biosynthetic heterogeneity to improve biosynthesis.

### 5.1. Single-cell phenotypic analysis technologies for heterogeneity monitoring and highly productive strain screening

The expression of DNA repair genes has been reported to exhibit phenotypic heterogeneity, which can lead to mutation rate variations between individual cells, a phenomenon called mutation rate heterogeneity [94,95]. Cells with high mutation rates are more likely to lose

their production phenotype due to mutagenesis in metabolic engineering. Exploring mutation rate heterogeneity can therefore help improve biosynthesis by controlling genetic heterogeneity. Recently, by combining microfluidics and time-lapse imaging, it is feasible to analyze the heterogeneity of DNA replication-related mutation rate in *E. coli* by imaging the endogenous expression of a fusion of yellow fluorescent protein (YFP) with MutL mismatch repair protein [96]. Mutagenesis is related not only to DNA replication but also to DNA damage repair, such as nucleotide excision, base excision and transcription-coupled repairs, among others [97]. Therefore, the development of a single-cell technology analyzing the total mutation rate is of great significance.

With the development of synthetic biology, an increasing number of product-responsive biosensors have been developed based on a transcriptional factor, riboswitch and aptazyme [98–100]. Product-responsive biosensors and product autofluorescences, combined with flow cytometry and microfluidics, have been widely used to monitor and analyze heterogeneity in biosynthesis performance during fermentation and screen highly productive phenotypes via directed evolution and high-throughput screening [11,101,102].

### *5.2. Combining multiple single-cell technologies for analyzing the mechanism of microbial heterogeneity*

Recently, single-cell transcriptome sequencing technologies that can be used for bacteria, such as prokaryotic expression profiling by tagging RNA in situ and sequencing (PETRI-Seq) [103], microbial split-pool ligation transcriptomics (microSPLiT) [104] and the poly (A)-independent multiple annealing and dC-tailing-based quantitative scRNA-seq (MATQ-seq) [105], have been developed. Single-cell transcriptome sequencing technologies combined with single-cell technologies analyzing the DNA replication-related mutation rate allow researchers to investigate mutation mechanisms at the single-cell level. Single-cell transcriptome sequencing technologies combined with single-cell technologies analyzing biosynthetic heterogeneity allow researchers to mine gene targets related to biosynthetic heterogeneity. In addition, the microfluidic-based single-cell isolation and culture system can perform time-resolved analysis of individual cells in accurately controlled environments [106]. When combined with single-cell isolation and culture systems, single-cell transcriptome sequencing and single-cell phenotypic analysis technologies can also be employed to explore the kinetics of microbial heterogeneity.

## 6. A combined workflow of IHP-RHR facilitated by novel single-cell technologies

Previous studies focused on only one side of microbial heterogeneity regulation, such as increasing heterogeneity in the strain-building step to construct diverse libraries of mutants or reducing heterogeneity in the fermentation process to improve bioproduction robustness. Therefore, we proposed a combined workflow of IHP-RHR to obtain highly

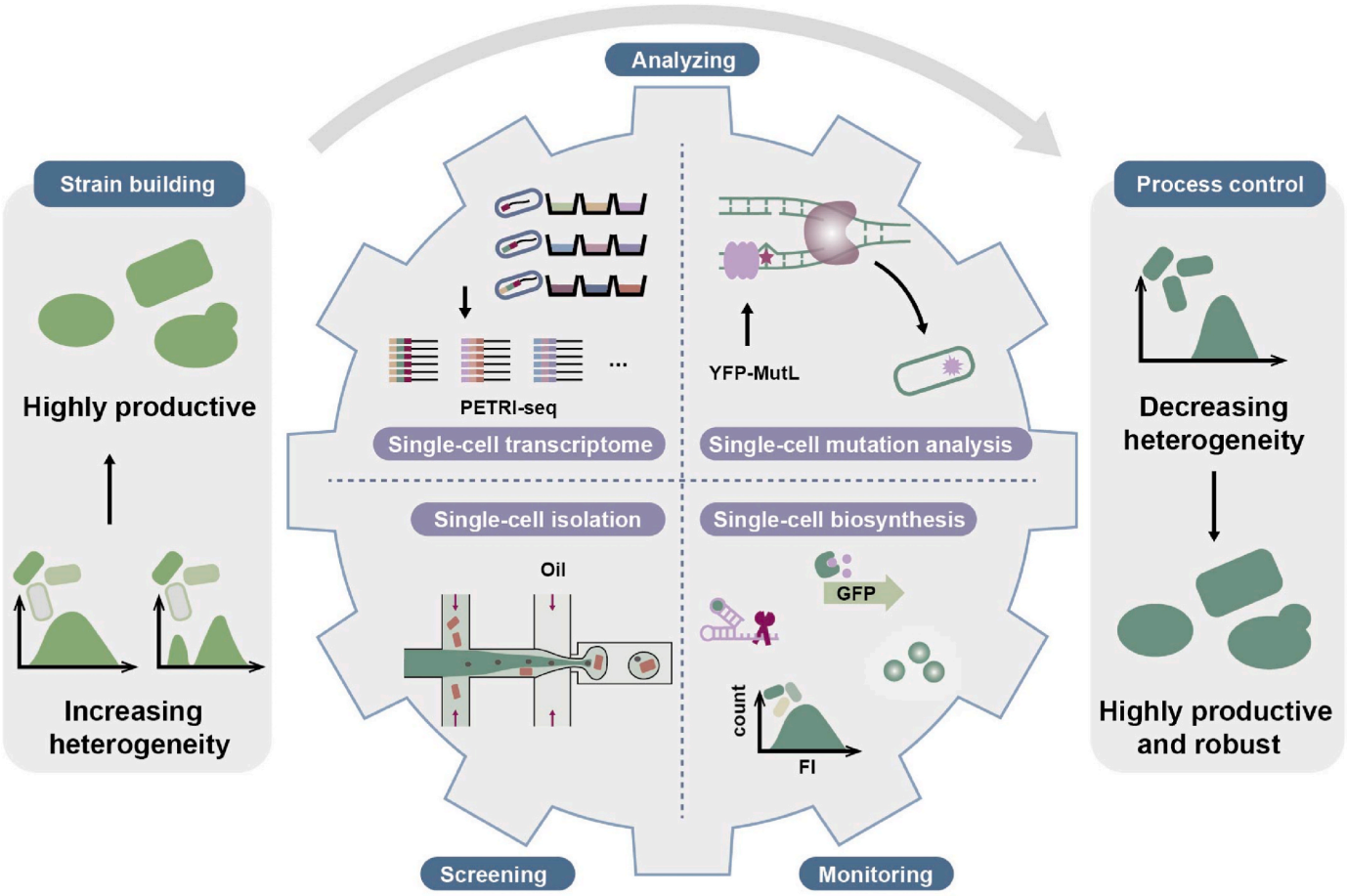


**Fig. 4.** A combined workflow, namely increasing genetic heterogeneity in the step of strain building to help in screening highly productive strains-reducing heterogeneity in the production process to obtain highly robust strains (IHP-RHR). It is promising to obtain highly productive and robust microbial strains using this combined workflow. In the strain-building step, increasing heterogeneity can provide a large library of variants. Then, flow cytometry and microfluidic-based single-cell analysis and isolation technologies can be employed to screen highly productive strains from the large library of variants. After this, reducing heterogeneity in the following fermentation process can help obtain highly productive and robust strains. In addition, novel single-cell analytical technologies can help explore microbial heterogeneity through monitoring and analysis, which can in turn provide novel targets for harnessing biosynthetic heterogeneity to push the workflow.

productive and robust microbial strains by integrating increasing/reducing heterogeneity (Fig. 4). Specifically, increasing heterogeneity using synthetic mutation regulation tools can provide a large library of variants in the strain-building stage. Then, highly productive strains can be screened out from the large library of variants by flow cytometry and microfluidic-based single-cell analysis and isolation technologies. In the conventional test of the highly productive strain, biosynthesis ability was accessed according to the total production of the population at a laboratory scale. However, biosynthesis heterogeneity between individual cells was neglected, which restricts the production robustness of industry-scale bioprocess. The development of single-cell technologies facilitated the measurement and analysis of biosynthesis heterogeneity between individual cells [92], and high-depth DNA sequencing and third-generation long-read technologies can help confirm whether genetic or non-genetic heterogeneity causes the biosynthesis heterogeneity [15,76]. Therefore, in the production process of highly productive strains, biosynthesis heterogeneity needs to be measured and analyzed. Then, appropriate tools and strategies to reduce heterogeneity can be applied to obtain highly productive and robust strains. In addition, exploring microbial heterogeneity by single-cell technologies can provide new insights into harnessing microbial heterogeneity, thereby pushing the workflow of IHP-RHR. Therefore, the IHP-RHR workflow facilitated by single-cell technologies has the potential to obtain highly productive and robust microbial strains by harnessing biosynthetic heterogeneity.

## 7. Prospects and future challenges

Significant progress has been made in increasing and reducing microbial heterogeneity. IHP-RHR workflow facilitated by single-cell technologies has the potential to obtain highly productive and robust microbial strains by harnessing biosynthetic heterogeneity. To achieve highly productive and robust bioproduction in the future, continuous efforts are essential to innovate or improve tools regulating heterogeneity and expand the availability of single-cell analysis technologies that can be used for microorganisms to further explore microbial heterogeneity. Although numerous tools have been developed to increase genetic heterogeneity, future work should focus on developing synthetic mutation regulation tools with higher mutation rates and broader mutation spectrum, which helps construct more diverse libraries of mutants within a shorter time. In addition, the results based on mutation accumulation and DNA sequencing have examined context-dependent mutation patterns, proving the existence of mutation bias [107,108]. Therefore, further exploring mutation bias and using computers to integrate the characteristics of easily mutated DNA and genetically stable sequences, tools can be developed to evaluate the genetic stability of DNA sequences, which can guide the selection and design of genetically stable gene sequences and genetic parts. In addition, high-depth DNA sequencing can detect genetic heterogeneity in early cultures, which should prevent the effect of early genetic heterogeneity on the scale-up of biosynthesis [15]. As mentioned above, metabolic coupling of growth with biosynthesis requires an in-depth understanding of complex metabolic networks. Therefore, genome-scale metabolic models can be used to discover new metabolic links that can achieve the coupling [109]. For coupling growth with biosynthesis based on product-responsive biosensors, developing novel biosensors and improving the performance of the existing biosensors based on a directed evolution platform help expand its applications [110].

Although combining single-cell transcriptome sequencing technologies with single-cell phenotypic analysis technologies allows for a systematic analysis of the mechanisms of microbial heterogeneity, it is insufficient to analyze only at the transcriptome level. Therefore, efforts can be made in the future to develop single-cell proteome and metabolome analysis technologies suitable for microorganisms. Furthermore, MS-based subpopulation proteomics analysis technologies can be used to explore microbial heterogeneity in combination with single-cell sorting technologies [111]. It can also be used to analyze differentially expressed proteins between different cell subpopulations, such as high- and low-productive cell subpopulations, allowing researchers to better understand the mechanisms of microbial heterogeneity in biosynthesis at the translation level, and filling the gap in microorganisms' single-cell proteome analysis technologies.

In conclusion, significant progress has been made in synthetic biology-derived tools and strategies to harness microbial heterogeneity. Novel single-cell technologies that can be used for exploring microbial heterogeneity. In addition, proper regulation of microbial heterogeneity is promising to obtain highly productive and robust strains. Therefore, a combined workflow of IHP-RHR facilitated by single-cell technologies should be considered in the future. However, the limitations in the mutation rates and spectrum of mutation regulation tools, availability of novel tools for measuring the genetic stability of DNA sequences, ubiquity of synthetic genetic circuits for reducing heterogeneity, and availability of single-cell proteome and metabolome analysis technologies for microorganisms are all issues that must be addressed.

## CRediT authorship contribution statement

**Yanting Cao:** Writing – review & editing, Writing – original draft, Validation, Investigation, Conceptualization. **Jianghua Li:** Validation, Investigation. **Long Liu:** Validation, Investigation. **Guocheng Du:** Validation, Supervision, Investigation, Funding acquisition. **Yanfeng Liu:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

## Declaration of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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