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Review

Advanced Technologies and Mechanisms for Yeast Evolutionary Engineering

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***In vitro* evolution is a powerful technique for the engineering of yeast strains to study cellular mechanisms associated with evolutionary adaptation; strains with desirable traits for industrial processes can also be generated. There are two distinct approaches to generate evolved strains *in vitro*: the sequential transfer of cells in the stationary phase into fresh medium or the continuous growth of cells in a chemostat bioreactor via the constant supply of fresh medium. In culture, evolutionary forces drive diverse adaptive mechanisms within the cell to overcome environmental or intracellular stressors. Especially, this engineering strategy has expanded to the field of human cell lines; the understanding of such adaptive mechanisms provides promising targets for the treatment of human genetic diseases and cancer. Therefore, this technology has the potential to generate numerous industrial, medical, and academic applications.**

Keywords: **Evolutionary engineering, *in vitro* evolution, yeast, adaptive mechanisms, gene therapy**

Introduction

Yeast is one of the most important micro-organisms in the scientific fields of biotechnology, biomedicine, and drug discovery [1]. Yeast cells have many biological advantages for industrial applications, such as high genetic amenability, low cost for cell culturing, and relatively quick cell division cycles [2]. There are two distinct engineering strategies to study the function of genes and develop industrial strains with improved capacity for stress resistance or production of value-added compounds [3]. First strategy uses gene manipulation techniques to permanently alter the genetic makeup through insertion, mutation, or deletion, which include recombination-mediated genetic engineering, clustered regularly interspaced short palindromic repeats, or error-prone polymerase chain reaction (PCR). The second approach employs *in vitro* evolution under the selective pressure of genetic or environmental stressors to identify strains with high tolerance to the stressful stimuli in an unbiased fashion.

While genetic engineering is a classical method to introduce targeted or intended genetic variation into a strain, evolutionary engineering exploits the interesting feature of yeast cells to rapidly adapt to genetic or environmental changes [4]. These adaptations to re-establish homeostasis and maintain viability from the acute stress conditions appear changes in diverse cellular pathways [5]. When these responses are not sufficient to protect cells from stress, yeast activate second-line adaptive mechanisms that introduce genetic changes to confer resistance to the stress [6]. Biotechnology typically use this adaptive laboratory evolution to biosynthesize new desirable products, improve production yields, or reduce costs in industrial processes [7]. The present review provides an overview of the evolutionary engineering of

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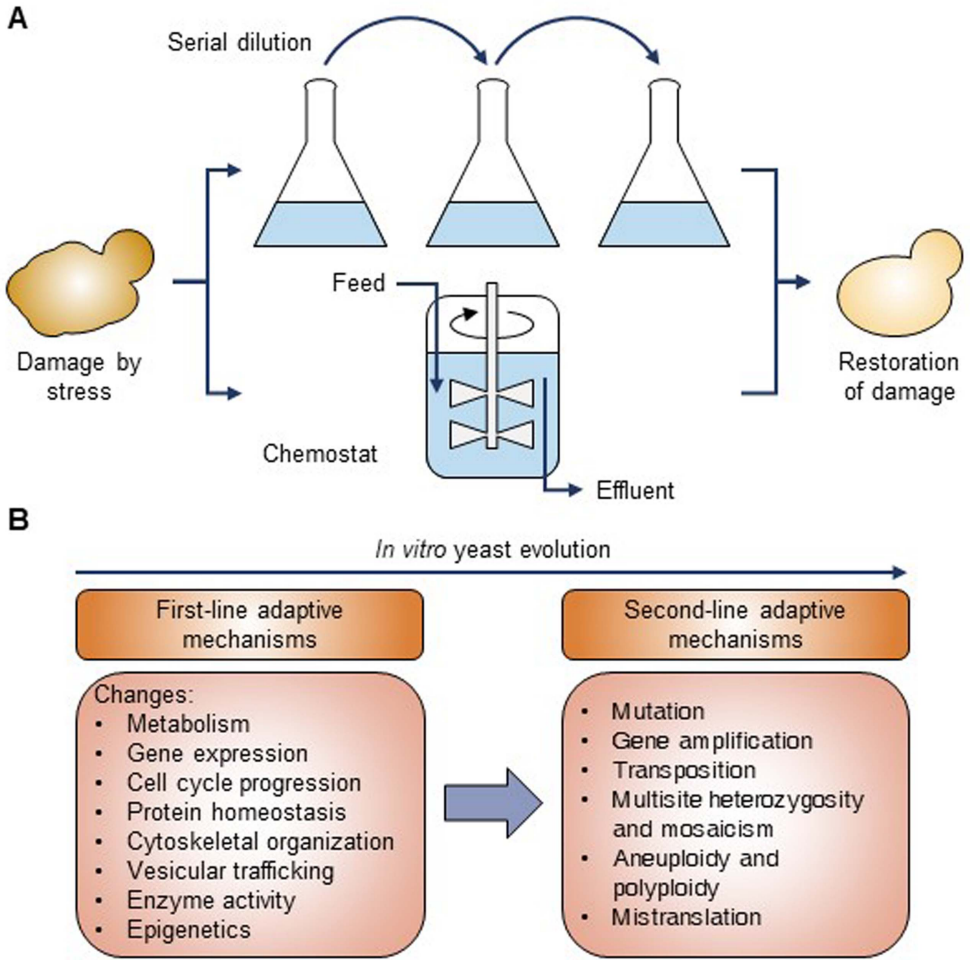

Fig. 1. Use of *in vitro* evolution methods to study adaptive mechanisms in yeast. (A) Two technical strategies of *in vitro* evolution: serial transfer and chemostat culture systems. Serial transfer propagates yeast in parallel serial cultures by doing sequential passages of cells (via dilution) once they have grown to stationary phase. Chemostat culturing keeps yeast cell numbers continuously growing in bioreactors while providing fresh medium. (B) Adaptive mechanisms of yeast during laboratory evolution culture.

yeasts for food/industrial biotechnology and the development of medical therapy.

Evolutionary engineering strategies

In vitro evolution is a general method to artificially induce cellular or genetic changes under specific growth conditions that are difficult to produce in nature, and therefore provides insights into the understanding of the molecular adaptation to the environmental change [7]. Usually, there are two main approaches in isolating evolutionary engineered cells that survive to the stress-induced conditions imposed by *in vitro* evolution [3] (Fig. 1A). First approach uses sequential passages of cells in shaking flasks or tubes to propagate yeast in parallel serial cultures. These cells are grown to stationary phase in a specific medium prior to being diluted and transferred into fresh medium. This process is repeated periodically (e.g., daily) until interesting phenotypes are acquired by the evolved cells. This method has the advantage of being low-cost and easy to operate and can also be automated to enable massively paralleled yeast cultures [8, 9]. The second approach is continuously growing cultures in chemostat bioreactors while providing fresh medium and keeping yeast cell numbers constant by reversely emitting equal amounts of effluents [10–12]. In contrast to the serial transfer culture, this technique maintains the physiological steady-state conditions of the cells and population densities and incorpo-

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rates relatively huge volumes of culture medium. Both strategies can precisely control the diverse culture conditions, such as nutrient supply, pH, temperature, oxygenation, or drug supplementation [13–16].

Methods to shorten the required time to achieve evolution enhance profits by saving resources and labor costs. Such methods including chemical mutagens, radiation, and genetic engineering can accelerate yeast evolution by increasing mutation frequencies in evolving cultures [3]. For instance, loss of Msh2 DNA mismatch protein function led to a 40-fold increase of mutation rate in the yeast genome, and the *mec1Δ tel1Δ* double mutant yeast strain exhibited chromosomal aneuploidy with large structural variations [17]. Furthermore, the expression of specific enzymes, such as the topoisomerase Cre recombinase, DNA glycosylase Mag1, or nuclease-deficient Cas9 (dCas9), can generate diverse genetic variation during evolutionary engineering [18–21].

Industrial applications of yeast evolutionary engineering

In biotechnology and food industries, yeast strains resistant to specific stresses are useful in enhancing the processivity, quantity, and quality of production for valuable materials, baking, brewing, and fermentation [22, 23]. For example, anaerobic starvation has been extensively investigated by nitrogen- or carbon-limited chemostat systems and has provided excellent advantages in the industrial production of bread, ethanol, and alcoholic beverages [24, 25].

The changes of endogenous energy metabolism to become tolerant to such stresses are driven by not only the control of specific genes but also diverse physiological changes [22, 26]. Several studies show the strong correlation between the change of intracellular trehalose concentration and the capability to resist heat and cold shocks [27]. Typically, continuous heat stress invokes a redistribution of catabolic and anabolic fluxes related to energy metabolism and increased ribonucleic acid (RNA) content [28, 29]. Other research found ultraviolet (UV) mutagenesis during 200 freeze-thaw cycles led to freeze-tolerant yeast strains that keep more gassing power during frozen dough storage [30]. Targeted *in vitro* evolution of the transcriptional regulator Stp15 led to reprogrammed gene transcription that conferred increased ethanol tolerance and conversion of glucose to ethanol in yeast [31].

Adaptive mechanisms of evolutionary engineered cells

Although extreme environmental or intracellular stressors often lead to cell death, those stresses that do not exceed a certain threshold are counterbalanced by rapid first-line protective mechanisms that confer survival [5] (Fig. 1B). Such responses can re-establish homeostasis and maintain viability by changes in metabolism, gene expression, cell-cycle progression, protein homeostasis, cytoskeletal organization, vesicular trafficking, and/or enzyme activity [32]. However, if a stress persists over time, cells often induce second-line adaptive mechanisms to promote genetic changes to maximize survival under continuous exposure [6]. These second-line adaptive responses need longer time to implement than the initial mechanisms. Therefore, *in vitro* evolution is employed to study the mechanism(s) in the laboratory in a feasible time-frame [33]. These adaptive strategies for the cell include mutation, gene amplification, transposition, multisite heterozygosity, mosicism (i.e., multiple sets of genetically different chromosomes), aneuploidy, polyploidy, and mistranslation. Many advances in bioinformatics and high-throughput genomic and proteomic analysis has allowed the identification and understanding of the molecular mechanisms behind stress-induced cellular evolution [34].

Epigenetic changes have also played a critical role in the adaptive response of yeast to stresses. Such epigenetic mechanisms allow rapid, reversible, and durable adaptations through histone or DNA modifications that alter the transcription, chromatin structure, nuclear organization, or pre-mRNA processing [35]. Also, prion-mediated regulation of protein state may contribute as triggers for adaptation without direct genetic change [36]. Although epigenetic regulation is important for both first- and second-line adaptive mechanisms and well established by the analytic approaches [e.g., chromatin immunoprecipitation (ChIP)-seq, ChIp-chip, etc.], the selection of suitable targets for epigenetic modifications and high material cost of experiments limits epigenetic analysis.

In vitro evolution-based new approach

Evolutionary engineering is a useful strategy for yeast cells to adapt to the stress of genetic defects or environmental changes. Most of these adaptations are genetic

variations in the expression of enzymes introduced by evolutionarily-conserved mechanisms. These mechanisms include higher rates of transcription that causes higher mutation frequencies due to promoting error-prone DNA polymerase activity, overwhelming the transcription-coupled DNA repair, aneuploidy stress, and error-prone nonhomologous end-joining DNA-repair pathway [37–40].

An example of a beneficial gene mutation is the loss of Ulp2 small ubiquitin-like modifier (SUMO) protease, which is involved in transcriptional regulation and chromosome cohesion [41–43]. In response to the acute loss of the Ulp2 enzyme, yeast cells undergo rapid induction of adaptive aneuploidy that counters the dysregulated SUMO system through the increased dosage of three genes *CCR4*, *CLN3*, and *CCW12*, and a cluster of small nucleolar RNA (snoRNA) genes, *SNR61*, *SNR55*, and *SNR57* [44–46]. With aneuploidy being deleterious to cell fitness [47], evolution over many cell generations results in chromosomal duplications being eliminated and creates favorable mutations in SUMO-ligating enzymes, *Ubc9*, *Uba2*, or *Aos1*, which reduce SUMO conjugation and suppress the growth defects of *ulp2Δ* cells [45]. Another case is the long-term exposure of yeast to heat or high pH that triggers advantageous gene mutations and alterations in gene expression [16].

Identification of the advantageous genetic change from *in vitro* evolution constitutes one of the greatest merits. This fascinating approach explores what the genetic manipulation is required to overcome the specific stressful conditions and can further suggests targets for therapy of human genetic diseases and cancers. Also, *in vitro* evolutionary engineering establishes new relationships between different cellular pathways. In the end, this strategy can still offer many insights for industrial, medical, and academic applications.

Discussion

Yeast evolutionary engineering method is widely and progressively used in the industrial applications to improve production of biosynthetic compounds. Recently, rapid advances in sequencing and gene-editing technologies have expanded the field of evolutionary engineering using yeast cells and therefore it enables to identify beneficial mutations and provide insight on the adaptive mechanisms [10, 11, 48, 49]. Furthermore, this strategy is also applicable to studying human diseases. Overtaking the culture of diverse cell lines often leads to appear beneficial mutations to adapt to specific culture conditions [50–54]. Therefore, this is a future-oriented research field and will offer a promising candidate for human gene therapy in the future.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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