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Biotechnology Methods – Mini-Review

Recent progress in adaptive laboratory evolution of industrial microorganisms

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Abstract: Adaptive laboratory evolution (ALE) is a technique for the selection of strains with better phenotypes by long-term culture under a specific selection pressure or growth environment. Because ALE does not require detailed knowledge of a variety of complex and interactive metabolic networks, and only needs to simulate natural environmental conditions in the laboratory to design a selection pressure, it has the advantages of broad adaptability, strong practicability, and more convenient transformation of strains. In addition, ALE provides a powerful method for studying the evolutionary forces that change the phenotype, performance, and stability of strains, resulting in more productive industrial strains with beneficial mutations. In recent years, ALE has been widely used in the activation of specific microbial metabolic pathways and phenotypic optimization, the efficient utilization of specific substrates, the optimization of tolerance to toxic substance, and the biosynthesis of target products, which is more conducive to the production of industrial strains with excellent phenotypic characteristics. In this paper, typical examples of ALE applications in the development of industrial strains and the research progress of this technology are reviewed, followed by a discussion of its development prospects.

Keywords: Adaptive laboratory evolution, Industrial strain breeding, Mutant library construction, Reverse metabolic engineering

Introduction

Adaptive laboratory evolution (ALE) is a method for the directed evolution of microorganisms and the selection of strains with superior traits from the evolving population by artificially simulating the mutation and selection processes in the natural environment under laboratory conditions (Sandberg et al. 2019; Lee & Kim, 2020). Microbial adaptive evolution is a representative technique for the study of biological evolution, commonly termed directed evolution, laboratory evolution, or domestication (Ryan et al. 2015). Microbial evolution is achieved through the application of artificial disturbances and control of the microbial growth environment. The source of evolution is the natural mutation of the microorganism itself or artificially induced mutations, while the direction of evolution is controlled by applying an artificial selection pressure. Similar to the natural environment, the driving force of evolution is the interaction between the microorganism and environmental factors, and the strains are selected for the desired target strains through individual competition and elimination of winners and losers over successive generations (Sandberg et al. 2019) (Figs. 1A and B). In contrast to metabolic engineering, ALE does not need to consider the intricate and intersecting metabolic networks of the cell, but only needs to design the corresponding selection pressure according to the target phenotype, which has the advantages of broad microbial applicability and practicality, making it easy to discover new mechanisms and achieve phenotypic optimization (Dragosits & Mattanovich 2013).

ALE can sustain rapid division of large numbers of cells, while the natural mutation rate and genome size of microorganisms can provide rich genetic diversity and constant enrichment of beneficial mutants (Gresham & Dunham 2014). Consequently, ALE allows strains to change certain phenotypic or physiological characteristics in a relatively short period of time, and basically does not affect other good traits of the strain (Wu et al. 2022). Because microorganisms have a short generation times and small size, large populations can exist in a small space, which helps to ensure reproducible experiments. Moreover, it is easy to preserve and compare the differences in characteristics between strains during evolution (Long & Antoniewicz 2018). ALE provides a powerful tool to study the factors affecting the phenotype, production performance, and genetic stability of industrial production strains, and has been successfully used in the field of microbial evolutionary research (Conrad et al. 2011). ALE not only enables laboratory studies of evolutionary processes, but it can also provide novel targets for metabolic engineering by identifying beneficial mutations associated with specific selection pressures or deleterious mutations that reduce production levels. In addition, it can also improve the physiological properties of engineered strains by compensating defects brought about by metabolic engineering modifications for specific phenotypes (Fig. 1C) (Sandberg et al. 2019). With the increasing popularity of low-cost, high-throughput sequencing technologies, the effective combination of genetic engineering and bioinformatics tools has greatly accelerated the identification and screening of key beneficial mutations in ALE-derived strains (Shendure et al. 2017). Analytical tools such as genomics, transcriptomics, proteomics, and metabolomics have likewise helped resolve the molecular mechanisms of adaptive evolution (Fig. 1D) (Sandberg et al. 2019). In addition, adaptive evolution holds great promise for obtaining information on

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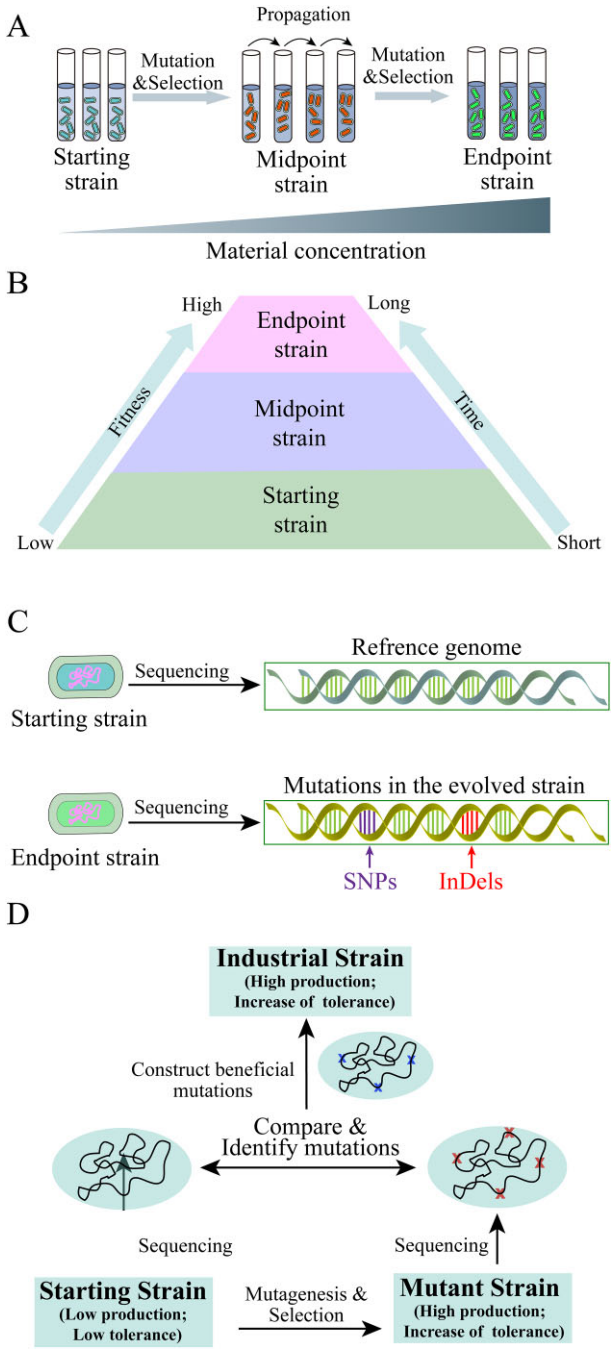

Fig. 1 Typical process of adaptive laboratory evolution (ALE). A: A schematic of the typical process of ALE. B: The relationship between evolutionary time and fitness. C: Genome sequencing of the evolved strains reveals the adaptive mutations that led to the improved phenotype. D: The typical progress of ALE applied to industrial microbial breeding.

genotype-phenotype relationships and for applications in medical and pharmaceutical research. ALE has become a powerful tool for microbiology research and is now generally used to screen excellent strains for industrial production that can satisfy specific needs. The application of ALE in the selection of industrial production strains is mainly focused on the following areas: (1) microbial metabolic pathway activation and phenotype optimization; (2) efficient utilization of specific substrates; (3) optimization of tolerance to toxic substances. ALE is often applied to model organisms, such as *Saccharomyces cerevisiae*, *Bacillus subtilis*, and *Escherichia coli*, because they can be easily manipulated and have mature fermentation protocols. However, non-model species that can use renewable raw materials for biofuel production have attracted increasing attention. Many non-model species are being explored for their metabolic strengths that can be leveraged to synthesize specific compounds, such as *Rhodococcus opacus*, *Yarrowia lipolytica*, and *Crypthecodinium cohnii* (Czajka et al. 2017). In this paper, we review the application of ALE in the selection and breeding of microorganisms for industrial production, dissect the problems of the evolutionary process as well as novel ideas for solving them, and discuss the future development of this technology.

Activation of metabolic pathways and phenotype optimization

Traditional metabolic engineering strategies include derepression of the target metabolic pathway, overexpression of genes encoding rate-limiting enzymes, increasing the supply of precursors. The goal of metabolic engineering is to modify the microbial metabolism for efficient synthesis of the target product, but this usually has a negative impact on cell growth. Moreover, for the biosynthesis of heterologous metabolites, it is necessary to construct exogenous metabolic pathways or activate native pathways, which need to be optimized to meet the increased precursor demand. ALE technology does not require in-depth understanding of complex microbial physiology, and can help obtain high-yielding strains by improving stress tolerance, optimizing metabolite production pathways, and improving metabolic fluxes (Pontrelli et al. 2018; Luo et al. 2019; Awasthi et al. 2022). ALE technology has been successfully used to optimize the growth rate of microorganisms (Hong et al. 2011; Yu et al. 2013; Pfeifer et al. 2017), alleviate growth defects (Aguilar et al. 2018; Thyer et al. 2018), investigate the variation of growth rate (Barrick et al. 2009; Lenski et al. 2015), as well as increase the biomass and product yield (Fong et al. 2005; Yu et al. 2013; Meyer et al. 2018; Godara & Kao 2020). Examples of microbial metabolic pathway activation and phenotype optimization using ALE are summarized in Table 1.

Hemansi *et al.* passaged *Kluyveromyces marxianus* JKH5 for 60 generations in medium containing inhibitors (acetic acid, furfural, and vanillin), and the specific growth rate of the final evolved strain was 3.3-fold that of the parental strain. Furthermore, the lag phase of the evolved strains was shortened by 56% under at 42°C, and the fermentation efficiency was increased by 80% (Hemansi et al. 2022). Garcia-Rios *et al.* evolved the starting strain *S. cerevisiae* L5 in sugar-based medium to improve fermentation and growth at high temperature. After 150 generations of continuous culture, the evolved strain L5EVO consumed 50% of the sugar in half the time compared to the initial strain (Garcia-Rios et al. 2021). Kim *et al.* obtained the evolved strain *E. coli* CY02 after 1 300 passages. Notably, its glycerol utilization increased by 46.8%, while the growth rate increased by nearly 40%, from 0.67 ± 0.03 to 0.94 ± 0.01 h^{-1} (Kim et al. 2022).

Tao *et al.* reported an evolved strain of *S. cerevisiae* that was able to generate 33.4 g/L of extracellular free fatty acids after 200 generations (Tao et al. 2018). Chen *et al.* developed a novel continuous ALE system (auto-CGSS) by integrating CRISPR/Cas9-facilitated *in vivo* mutagenesis with real-time measurement of cell growth and online monitoring of tryptophane-mediated fluorescence intensity. This strategy yielded a promising engineered strain that could produce 38.77 g/L tryptophan (Chen et al. 2021). Reyes *et al.* used *S. cerevisiae* GSY1136 as a starting strain and obtained an evolved strain by short-term evolution using a cyclic hydrogen peroxide stimulation protocol. The carotenoid production of the evolved strain was increased three-fold, reaching a yield

Table 1. Representative examples of the activation of microbial metabolic pathways and phenotype optimization by ALE.

Evolved strain	Evolution/selection conditions	Characteristics of evolved strains	References
S. cerevisiae Y&Z053	The evolving population was subcultured for 45 days in medium with gradually increasing glucose concentrations (from 0.5 to 2%) and decreasing ethanol concentrations (from 2 to 0.5%). Finally, 2% glucose was used as the only carbon source for 50 days of ALE	*S. cerevisiae* Y&Z053 can produce 33.4 g/L free fatty acids.	(Tao et al. 2018)
E. coli S028	Continuous mutagenesis was performed using CGSS at 30°C.	This evolved strain could produce 38.77 g/L of tryptophan with a yield of 0.164 g/g.	(Chen et al. 2021)
E. coli DSM01	Continuous evolution was carried out for 40 days in medium containing sodium acetate.	The parental strain could not grow in acetate-containing medium, but the evolved strain had a maximum growth rate of 0.061 ± 0.001 h^{-1}.	(Seong et al. 2020)
B. coagulans WT-03	After 15 s of ARTP mutagenesis and 40 days of ALE in media with 0.3% bile salt (pH gradually decreasing from 6.2 to 2.5).	Tolerance to pH 2.5 and 0.3% bile salts with a survival rate of 22.4%.	(Liu et al. 2020)
E. coli GS-2-4	Gradually increase the concentration of 4HPAA (from 15 to 35 g/L) for adaptive evolution.	The 4HPAA titer was 25.42 g/L at 88 h, which was 127.3% higher than that of the parental strain.	(Shen et al. 2022)
E. coli W3110 GE Δ*ung*	Gradually increase the concentration of ethanol and isopropanol in a 1% gradient	The tolerance levels of the expression mutants for ethanol and isopropanol increased by 8% and 3%, respectively.	(Eom et al. 2022)
P. tricornutum ALE-Pt1	A total of 30-cycles of continuous culture over 240 days was conducted at a 30 g/L glucose concentration gradient combined with alternating temperatures of 10°C or 20°C.	Biomass increased from 0.84 to 2.19 g/L, lipid content accounted for 33.76% of dry cell weight, with 31.18% of PUFAs in total fatty acids	(Wang et al. 2022)
K. marxianus JKH5 C60	Serially passaged for 60 generations at 42°C in a medium containing 3 g/L acetic acid, 1 g/L furfural, and 1 g/L vanillin.	Growth rate increased from 0.03 to 0.13 h^{-1} and the evolved strain had 5 times the biomass of the starting strain.	(Hemansi et al. 2022)
E. coli CY02	Adaptive evolution over 1300 generations in M9 minimum medium supplemented with 0.2% (V/V) glycerol.	Improved the final GABA titer and specific productivity by 3.9- and 4.3-fold, respectively.	(Kim et al. 2022)
Z. mobilis Z198	Adaptive evolution was conducted in medium based on corn stover hydrolysate by successive transfer every 24 h for 198 days.	The tolerance to the most toxic phenolic (vanillin) was significantly increased by 6.3-fold, the ethanol productivity reached 0.91 g/L·h, and the ethanol concentration increased by 21.6%.	(Yan et al. 2021)
S. cerevisiae L5EVO	Continuous passaging for 150 generations at 40°C and 100 rpm.	The maximum biomass was 2-fold that of the initial strain, and the time required to consume 50% of the sugar was cut in half.	(Garcia-Rios et al. 2021)
C. cohnii E-D	First increasing the concentration of sethoxydim (from 10 to 60 μM) for 525 days, and then increasing the concentration of sesamol (from 0.5 to 2 mM) for 300 days.	After ALE with 1 mM sesamol, the lipid and DHA production rates of the evolved strain reached 0.046 g/L·h and 0.025 g/L·h, respectively.	(Diao et al. 2019)

of up to 18 mg/g [DCW] (Reyes et al. 2014). In order to improve the production of polyunsaturated fatty acids (PUFAs) by natural microalgal strains, a novel two-factor adaptive evolution scheme was developed (Wang et al. 2022). After 90 generations of evolution, the lipid content reached 33.76% of dry cell weight (DCW), and PUFAs accounted for 31.18% of total fatty acids (TFAs). Diao *et al.* developed a new method called 'chemical modulator based adaptive laboratory evolution' (CM-ALE) to improve lipid and docosahexaenoic acid (DHA, 22:6n-3) production. They used *C. cohnii* ATCC 30556 as the starting strain and obtained the evolved strain E–D through a two-step CM-ALE process spanning 825 days. By adding 1 mM sesamol, the yield of lipid and DHA was doubled to 0.046 and 0.025 g/L·h, respectively (Diao et al. 2019). Caspeta *et al.* used an ALE strategy to screen heat-resistant strains of *S. cerevisiae* CEN.PK113-7D. The evolved strain was obtained by successive passages for over 300 generations at 39.5 ± 0.3°C, with a 57% increase of the specific growth rate, as well as 60 and 30% increases of ethanol and glycerol production at 40°C, respectively (Caspeta et al. 2014).

Efficient utilization of substrates

Efficient substrate utilization is essential to achieve efficient metabolite synthesis. There is a direct link between microbial adaptations and the ability to efficiently absorb and metabolize growth-limiting nutrients during fermentation, and therefore, adaptive evolution through the design of selective culture conditions can easily screen for genetic mutations that contribute to substrate utilization. In addition, expanding the range of substrate utilization enable microbes to transform substances that are difficult to utilize or have growth inhibitory effects (Ji et al. 2011; Plácido & Capareda 2016; Jang et al. 2019). Typical examples of substrate utilization improved by ALE are summarized in Table 2. *S. cerevisiae* is widely used in the fermentation industry,

Table 2. Representative examples of enhancing the effective utilization of substrates by microorganisms using ALE.

Evolved strain	Utilization of substrates	Evolutionary screening conditions	Characteristics of evolved strain	References
S. cerevisiae	18 g/L galactose concentration	Continuously increasing the galactose concentration in the medium (from 20 to 80 g/L).	Ethanol yield increased from 8.9 g/L to 20 g/L with an ethanol yield coefficient (Y_{EtOH}) of 0.44 g/g.	(Sunwoo et al. 2019)
S. cerevisiae CEN.PK113	6% glycerol	Continuously subcultured for 60 rounds in synthetic glycerol medium.	Specific growth rate increased from 0.125 h^{-1} to 0.164 h^{-1}.	(Ho et al. 2017)
K. marxianus MTCC 1389	200 g/L lactose	Serial cultivation was carried out for 750 passages in medium containing 200 g/L lactose.	Ethanol titer increased by 17.5%, to 79.33 g/L.	(Saini et al. 2017)
C. necator H16	0.5% glycerol	Subculture in media with 0.5% glycerol.	Growth rate increased 9.5-fold to 0.20 h^{-1}.	(Gonzalez-Villanueva et al. 2019)
C. glutamicum MX-11	15 g/L methanol and 4 g/L xylose	Continuous culture in CGXII minimal medium.	The co-utilization ratio of methanol and xylose reached 7.04:1, the specific growth rate increased 3.20-fold.	(Wang et al. 2020)
Z. mobilis ATCC AD50	Glucose and xylose	ALE was carried out for 200 days in three stages with xylose concentrations of 3%, 5%, and 10%; 10, 20, and 20 passages of culture were performed, respectively.	The maximum biomass of the evolved strain increased 2-fold (OD_{600} of 8), the ethanol titer increased from 12.65 g/L to 49 g/L, while the utilization rates of glucose and xylose reached 1.24 and 1.34 g/g·h, respectively.	(Sarkar et al. 2020)
G. oxydans RM7	21.8 g/L xylose	Strains were alternately transferred in media with and without inhibitor (containing 21.8 g/L of xylose) for 420 days of continuous evolution.	The conversion rate of xylose was 4.1-fold higher than in the starting strain.	(Jin et al. 2019)
C. glutamicum $WMB2_{evo}$	10 g/L D-glucose	Using 20g/L D-xylose as the sole carbon source, evolution was carried out continuously for 35 generations at 30 °C and 130 rpm.	Growth rate tripled to 0.26±0.01h^{-1}, and the specific D-xylose uptake rate reached 5.7±0.1 mmol/g·h.	(Radek et al. 2017)
S. cerevisiae BN-91A	20 g/L mannitol	Nearly 1500 hours of evolution by serial dilution culture with mannitol as the sole carbon source until the specific growth rate doubled.	Mannitol assimilation rate increased 3-fold and assimilation rate reached 0.164 g/L·h.	(Zhu et al. 2022)
C. glutamicum ATCC 13032	Methanol and xylose	Continuous evolution for 206 days at 30°C and 220 rpm in media containing 4 g/L methanol and xylose, respectively.	The specific growth rate was increased 20-fold, with methanol and xylose co-utilization at a high molar ratio of 3.83:1.	(Tuyishime et al. 2018)

and its strong fermentation capacity and inherent ethanol tolerance make it a constant focus of research. Researchers often use ALE to enhance the substrate utilization efficiency and thereby improve the ethanol yield. Kawai *et al.* used *S. cerevisiae* W303-1B as the starting strain, and passaged it continuously for 70 generations in a medium with glycerol as the main carbon source. This ALE approach improved the glycerol assimilation capacity of the yeast, while the growth rate of the evolved strain reached 0.14 h^{-1} (Kawai et al. 2019). Zhu *et al.* grew *S. cerevisiae* BN-00 in liquid synthetic medium with mannitol as the sole carbon source. After nearly 1 500 h of ALE, strain BN-91A with good mannitol assimilation ability was obtained. The mannitol assimilation rate of BN-91A was three-fold higher than that of BN-01A, reaching 0.164 g/L·h (Zhu et al. 2022). Furthermore, Sunwoo *et al.* adaptively evolved *S. cerevisiae* CEN.PK2-1 to increase the utilization of galactose, thereby increasing the production of ethanol, with the product titer increasing from 8.9 to 20 g/L, corresponding to an ethanol yield of 0.44 g/g (Sunwoo et al. 2019). In addition, *S. cerevisiae* cannot metabolize xylose or other pentoses for fermentation, but there have been numerous studies to construct engineered strains that co-utilize these compounds (Millan et al. 2020; Promdonkoy et al. 2020; Sarkar et al. 2020). Some scholars have attempted to construct xylose-utilizing *S. cerevisiae* strains using metabolic engineering methods, but ultimately no high-yielding strains that can convert xylose to ethanol was obtained, and neither the wild-type strains nor the existing engineered strains could use xylose as a substrate under anaerobic conditions. Fortunately, ALE tools could address this problem and improve the utilization of xylose. Klimacek *et al.* adapted *S. cerevisiae* BP10001 to anaerobic growth on xylose, achieving a 40-fold increase of the OD_{600} (from 0.12 to 4.8) after 91 days of continuous evolution to obtain strain IBB10A02, which was then passaged for another 56 rounds to obtain strain IBB10B05. Compared with IBB10A02, its growth rate increased by 80% to 0.056h^{-1}, and the ethanol yield reached 0.35 ± 0.02 g/g xylose (Klimacek et al. 2014).

ALE was also used to improve the utilization of xylose by other strains. Radek *et al.* developed an automated and miniaturized ALE method based on repeated batch culture in a microtiter plate (Radek et al. 2017). This method enabled *Corynebacterium glutamicum* pEKEx3-xylXABCDCc to utilize D-xylose via the Weimberg (WMB) pathway. The final evolved strain was able to grow with D-xylose as the sole carbon source, and the growth rate reached 0.26 h^{-1}. Kim and Lee evolved a natural xylose-fermenting *Pichia stipitis* strain to utilize both cellobiose and xylose. The ethanol yield of the evolved strain increased from 0 to 0.4 (g ethanol/g cellobiose) (Kim & Lee 2019). Sarkar *et al.* used the recombinant xylose fermenting strain *Zymomonas mobilis* ZW658 as the starting strain, and carried out three-stage ALE experiments for a total of 200 days (Sarkar et al. 2020). The maximum biomass of the evolved strain increased twice (OD_{600} of 8), while the utilization rates of glucose and xylose increased to 1.24 and 1.34 g/g·h, respectively. Jin *et al.* conducted a 420-day ALE experiment, which increased the xylose utilization efficiency of the strain *Gluconobacter oxydans* DSM 2003 4.1-fold, while arabinose, mannose, and galactose could also be completely converted into the corresponding sugar acids within 72 h (Jin et al. 2019). Many similar studies have been conducted with bacteria such as *E. coli* (Cordova et al. 2016; Sandberg et al. 2017). Tuyishime *et al.* evolved methanol-dependent *C. glutamicum* MX-10 for 206 days, and the evolved strain co-utilized methanol and xylose at a significantly improved ratio of 3.83:1, while the specific growth rate increased from 0.0006 to 0.0121 h^{-1} (Tuyishime et al. 2018). Zhang *et al.* used adaptive evolution of *B. subtilis* 168 to obtain the mutant strain E72 that efficiently utilized xylose with a maximum specific growth rate of 0.445 h^{-1} (Zhang et al. 2015). González-Villanueva *et al.* adapted *Cupriavidus necator* H16 to mineral salt medium containing 0.5% glycerol and screened the evolved strain v6C6 that could utilize both gluconate and glycerol, with a 9.5-fold faster specific growth rate in glycerol than the wild-type strain. In addition, the evolved strain grew faster on the mixed gluconate-glycerol carbon source than on gluconate alone (Gonzalez-Villanueva et al. 2019).

Increasing the tolerance to toxic substances

Under the stress of a complex growth environment, specific inhibitors or toxic products can significantly inhibit the growth of industrial strains. Studies have shown that ALE technology can overcome adverse effects and enhance cellular robustness, allowing microbial strains to both tolerate unfavorable growth environments and grow rapidly under favorable culture conditions (Ai et al. 2016; Pereira et al. 2019). Researchers have investigated the adaptive response mechanisms of strains to stress factors such as temperature (Deatherage et al. 2017), inhibitors (Henson et al. 2018; Wang et al. 2018; Xu et al. 2018), osmotic pressure (Tilloy et al. 2014; Winkler et al. 2014), pH (Fletcher et al. 2017), UV irradiation (González-Ramos et al. 2016), and nutritional factors (summarized in Table 3).

Some beneficial compounds cannot be synthesized in large quantities in the host due to their toxicity. ALE technology can enhance the tolerance of the strain, thereby increasing the final titer of toxic products. Mundhada *et al.* acclimated *E. coli* Q1 by gradually increasing the L-serine concentration in the medium. The evolved strains obtained after 45 days had significantly enhanced tolerance to L-serine, and after 50 h of fermentation with glucose as the carbon source, the yield of L-serine reached 37 g/L, corresponding to a conversion rate of 24% (Mundhada et al. 2017). Cavero-Olguin *et al.* used *Propionibacterium acidipropionici* DSMZ4900 as the starting strain, and the evolved strain was able to tolerate 30 g/L propionic acid (PA). Moreover, it was found that the production rate was improved from 0.17 to 0.52 g/L·h, while the final PA titer was improved from 8.72 to 16.8 g/L (Cavero-Olguin et al. 2021). Niu *et al.* evolved strain YZ-3 from *E. coli* BW25113 (PT5-dxs) by gradually increasing the concentration of pinene in the medium. YZ-3 was able to produce 7.3 ± 0.2 mg/L of pinene, which was 31% higher than the yield of BW25113 (PT5-dxs) (Niu et al. 2018). Elbakush and Güven. obtained osmotolerant and ethanol tolerant *S. cerevisiae* strains by successive batch fermentation. Notably, the ethanol yield of the evolved strain was increased to 2.03 g/L·h, while it was only 1.65 in the wild type (Elbakush & Güven 2021). Li *et al.* adaptively evolved *S. cerevisiae* SyBE005 (E7) by gradually increasing the concentration of inhibitor mixtures (phenol, phenolic acid, and acetic acid). The ethanol yield of the evolved strain E7-403 was up to 80% higher than that of the starting strain in the presence of the inhibitor cocktail (Li et al. 2019).

In a novel approach, Zhu *et al.* developed an ALE strategy based on stress-induced mutagenesis (SIM) in non-dividing cells (Zhu et al. 2014). After 9 months of ALE, minimum inhibitory concentration of butanol of the evolved strain *E. coli* SMB07was increased to 13 g/L, compared to 9.5 g/L in the starting strain. On this basis, they applied further ALE of SIM to strengthen its osmotic and heat tolerance, and ultimately obtained strain SMB500 that could tolerate 95 g/L NaCl and 50°C, respectively. Studies have shown that Atmospheric Pressure Room Temperature Plasma (ARTP) is an efficient mutagenesis strategy to enhance the probiotic properties of *Bacillus coagulans* in combination with ALE, resulting in

Table 3. Representative examples of improving microbial tolerance to toxic products by ALE.

Evolved strain	Toxic substance	Evolutionary screening conditions	Characteristics of evolved strain	References
E. coli SMB07	Butanol and NaCl	Progressively increasing concentrations of butanol and NaCl in the medium.	Tolerant to high concentrations of butanol (13 g/L), NaCl (95 g/L), and high temperature (50°C).	(Zhu et al. 2014)
E. coli ML115	Octanoic acid	Octanoic acid was periodically increased by 10 mM up to 30 mM in MOPS medium. The evolution lasted a total of 714 h.	The evolved strain produced 180±30 mg/L octanoic acid (5.6-fold the titer of the starting strain).	(Royce et al. 2015)
S. cerevisiae	Ethanol	Gradually increased the glucose concentration (from 50 to 150 g/L) in continuous batch fermentation.	Tolerated 61 g/L of ethanol, and the production rate of ethanol reached 2.03 g/h, which was 7.7-fold higher than in the starting strain.	(Elbakush & Güven 2021)
P. acidipropionici	Propionic acid	Gradually increased the concentration of propionic acid in the medium (from 10 g/L to 40 g/L).	Evolved strain tolerated 30 g/L of propionic acid. Propionic acid production rate, concentration and strain growth rate were all improved. (respectively: 0.17 to 0.52 g/L·h, 8.72 to 16.8g/L, 0.16 to 0.23g CDW/L·h)	(Cavero-Olguin et al. 2021)
E. coli M20	Isobutyl acetate	Evolved by cultivating in M9P medium containing 1.5 g/L IBA and gradually increasing the concentration of IBA.	The product titer increased 2.8-fold to 3.4 g/L.	(Matson et al. 2022)
S. cerevisiae E7-403	Phenol, furfural and acetic acid	Mixed inhibitor concentration gradually increased, grown at 30 °C and 200 rpm.	The ethanol yield of E7-403 was up to 80% higher than in the starting strain.	(Li et al. 2019)
E. coli ALE-5	Serine	Evolved for 45 days using gradually increasing serine concentrations (from 3 to 100 g/L) in the medium.	The evolved strain was able to tolerate 50g/L serine, and the product titer reached 37.3 g/L.	(Mundhada et al. 2017)
E. coli YZ-3	Pinene	Cultures were continuously passaged into fresh LB medium containing pinene (0.5% to 2%).	Increased pinene tolerance to 20 g/L, and increased the product titer by 31% to 7.3 mg/L.	(Niu et al. 2018)

strains with potential industrial applications. Liu *et al.* used *B. coagulans* WT-03 as the starting strain, and exposed it to ARTP to induce mutations, which was combined with ALE to improve the performance of probiotics. After 15 s of ARTP exposure and 40 days of ALE, the mutant strain artp-aleBC15 was able to tolerate pH 2.5 and 0.3% bile salts (Liu et al. 2020). Tian *et al.* started with *S. cerevisiae* ET008, mutated it using ARTP, and performed laboratory evolution (Tian et al. 2020). After 15 rounds of mutation and 90 days of laboratory evolution, the strain ET008-c54 exhibited a 10-fold higher survival rate at low pH. Shen *et al.* used a combination of ARTP and ALE to improve the yield of 4-hydroxyphenylacetic acid (4HPAA) and product tolerance in *E. coli* (Shen et al. 2022). The growth performance of the obtained evolved strains Gs-2–4 and Gs-2–12 increased by 23.8 and 26.5%, respectively, while the final concentration of 4HPPA increased by 127.3 and 118.7%, respectively. Smith and Liao. used *E. coli* JCL16 as the starting strain and performed two rounds of N'-nitro-N-nitrosoguanidine (NTG) mutagenesis to construct a mutant library, which was then subjected to ALE to obtain the evolved strain NV3, which exhibited higher substrate tolerance (8 g/L isobutanol) and higher yield (21.2 g/L isobutanol) than the parental strain (Smith & Liao 2011).

Royce *et al.* steadily increased the concentration of octanoic acid in the medium during 714 h of ALE, and the final evolved strain *E. coli* LAR1 was able to tolerate 30 mM octanoic acid, while its yield increased from 32 ± 7 to 180 ± 30 mg/L (Royce et al. 2015). Matson *et al.* performed ALE experiments to enhance the tolerance of *E. coli* JCL260 to isobutyl acetate (IBA). The final evolved strain was able to tolerate 3.4 g/L of IBA, which was 2.8-fold

higher than that of JCL260 (Matson et al. 2022). Overbeck *et al.* constructed *Lactobacillus casei* mutants by knocking out the *mutS* gene of *L. casei* 12A. The obtained mutant library was subjected to ALE for 100 days, and the final evolved strain could grow well at pH 2.5, with a 10-fold higher survival rate than the wild-type strain (Overbeck et al. 2017). Wang *et al.* conducted evolutionary experiments starting with the industrial methanol-dependent strain *C. glutamicum* MX-11, and the evolved strain was tolerant to 203.75 mmol/L methanol with a growth rate of 0.052 h^{-1} (Wang et al. 2020). Yan *et al.* performed 198 days of ALE on *Z. mobilis* 8b to improve its tolerance to phenolics in corn stover hydrolysate and ethanol fermentation capacity (Yan et al. 2021). The results showed that the conversion of the most toxic phenolic compound (vanillin) was significantly increased by 6.3-fold, the production rate of cellulosic ethanol reached 0.91 g/L·h, and the yield was increased by 21.6%. Seong *et al.* successfully obtained *E. coli* SBA01 through 40 days of continuous evolution (Seong et al. 2020). This strain consumed 2.14 g/L of acetate in 48 h, an 7.6-fold increase over the parental strain, while also being able to tolerate 250 mmol/L acetate, with a growth rate of 0.049 ± 0.001 h^{-1}. Eom *et al.* developed a novel *in vivo* mutagenic enzyme that directly modifies genomic DNA. They then gradually increased the concentration of ethanol in a 1% gradient, and the final tolerance level of mutant cells reached 8%, while that of non-mutant cells was 6.85% (Eom et al. 2022). Liu *et al.* used sub-culture in fermentation supernatant as the selection pressure and gradually increased its concentration (from 30 to 90%) for 840 days of ALE to obtain the evolved strain *C. cohnii* FS280. This strain exhibited greatly improved resistance to inhibitors form the fermentation supernatant and higher DHA production. When cultured in 90% supernatant for 72 h, the growth and DHA productivity of the evolved strain increased by 161.87 and 311.23%, respectively (Liu et al. 2022).

Emerging DNA technologies provide novel tools for ALE library construction

In order to avoid the accumulation of lethal mutations, microorganisms have strict mismatch repair mechanisms that eliminate wrong bases inserted during replication and recombination events, which reduces the natural mutation rate to a very low level. For instance, the spontaneous background mutation rates of *E. coli*, *S. cerevisiae*, and *Y. lipolytica* are 1.1×10^{-6}, 1.0×10^{-6}, and 1.3×10^{-6}, respectively (Czajka et al. 2020). Consequently, ALE faces a practical problem because the evolutionary period usually lasts long, ranging from months to years. This increases the risk of contamination in passaging or continuous culture processes, which in turn leads to experimental failure (Long & Antoniewicz 2018). Accordingly, genetic diversity limits the possibilities of adaptive evolution (Wang et al. 2018; Yang et al. 2018). To overcome this bottleneck, many genetic diversification techniques were developed to enhance the mutation frequency (summarized in Table 4).

There are several traditional ways to construct gene libraries. Random mutations can be induced by chemical mutagenesis, physical mutagenesis, and genomic recombination. Alkylating compounds, base analogues, and genotoxic antibiotics are often used in chemical mutagenesis (D'Souza et al. 2019; Zhou & Alper 2019). Smith and Liao combined NTG mutagenesis with ALE to obtain isobutanol-producing *E. coli*, and the enhanced isobutanol production phenotype of the strain was found to be derived from an *ldhA* (R118C) mutation (Smith & Liao 2011). Ang *et al.* improved the mutation efficiency of *E. coli* by paired combination of classical base analog mutagens. The combination of the adenine analog 2-aminopurine (2AP) and cytidine analog zebularine (ZEB) increased the mutation frequency of the *rpoB* gene 35-fold, reaching 53 000 × 10^8, which was higher than that of each mutagen alone, showing a significant synergy (Ang et al. 2016). Common physical mutagenesis methods include X-rays, γ-rays, ultraviolet (UV) light, ARTP, and atmospheric pressure glow discharge (APGD) (D'Souza et al. 2019). They can lead to base conversions, insertions and deletions of bases, directly changing the DNA sequence. Lv *et al.* used ARTP to produce more than 1 600 mutant strains, among which they screened the mutant *C. cohnii* 16D with a higher DHA yield. Notably, the productivity and DHA yield of this train in batch fermentation were increased by 70 and 30% (to 57.7 mg/L·h and 52.0 mg/g), respectively (Lv et al. 2020). In recent years, ARTP has been widely used to construct mutant libraries, and many researchers obtained improved strains by combining ARTP and ALE methods (Zhang et al. 2014; Liu et al. 2020; Tian et al. 2020). Jiang *et al.* proposed a novel heterologous biosynthetic strategy that combines ARTP-induced mutagenesis and hydrogen peroxide-driven adaptive evolution. They constructed the parental strain AX0 for mutagenesis and adaptive evolution based on *S. cerevisiae* SyBE_SC1402. After three cycles of ARTP and two rounds of H_2O_2 treatment, three different mutants (AX13, AX14, AX15) were finally obtained, and the astaxanthin concentration was increased to 59.36, 63.22 and 65.93 mg/L, respectively (Jiang et al. 2020). Genome Shuffling produces multiparental strains through the fusion of protoplasts. Lin *et al.* combined genome recombination and adaptive evolution to obtain a hybrid strain capable of glucose-xylose co-fermentation at high temperature (Lin et al. 2019). The starting strain SSP was obtained by performing three rounds of genome recombination, followed by a five-month adaptive evolution experiment. The final evolved strains X2 and X5 produced 20 and 50% higher ethanol yields than the parental strain at 40°C, respectively. Although genome shuffling can increase genetic diversity, it is extremely difficult to construct initial strains, which limited its broader applications.

Traditional physicochemical mutagenesis was also found to be unsustainable for increasing mutation rates in ALE experiments. As a consequence, several feasible strategies were developed to continuously maintain high mutation rates during the ALE process, such as Helicase-AID (Wang et al. 2021), blocking DNA mismatch repair (Badran & Liu 2015; Csorgo et al. 2016), Genome Replication Engineering Assisted Continuous Evolution (GREACE) (Luan et al. 2015), Restriction-Modification (R-M) system (Yoko et al. 2011; Bai et al. 2018), and CRISPR-enabled trackable genome engineering (CREATE) (Chen et al. 2020). Wang *et al.* designed and constructed a novel genomic random mutation system called DnaB-AID. It enables C to T editing of random genomic loci in *E. coli* and *S. cerevisiae*, resulting in 2.5×10^3 and 1.68×10^3-fold higher mutation rates than the wild type, respectively (Wang et al. 2021). The microbial DNA Mismatch Repair (MMR) mechanism is designed to specifically repair the microbial DNA mismatches between new and formed bases in DNA strands during replication (Reyes et al. 2015). The literature demonstrates that disruption of the MMR system can maintain a high mutation rate in the cell. Luan *et al.* inactivated the genomic MMR system manipulator MutSL of *C. acetobutylicum*, and the natural mutation rate increased up to 250-fold. Compared with the control strain, the engineered strain showed better survival ability in the butanol tolerance evolution experiment, and the evolution rate was greatly increased (Luan et al. 2013). In addition, Luan *et al.* developed a GREACE method based on 'mutagenesis coupled-with

Table 4. Representative examples of mutant library construction for ALE.

	Strategy and description	Characteristics of evolved strains	References
Genetic diversification by introducing non-targeted mutations	Traditional physical and chemical mutagenesis by adding chemical reagents or by X-ray, γ-ray, ultraviolet (UV), Atmospheric and Room Temperature Plasma (ARTP) and Atmospheric Pressure Glow Discharge (APGD) treatment	The combination of the adenine analog 2-aminopurine (2AP) and cytidine analog zebularine (ZEB) increased the mutation frequency of the *rpoB* gene 35-fold to $53{,}000\times10^{8}$.	(Ang et al. 2016)
	Increased mutation rate and DNA exchange by recursive protoplast fusion (genome shuffling) with multiple parental strains for combinatorial phenotypes.	After three rounds of genome recombination and 5 months of ALE, evolved strains X2 and X5 produced 20 and 50% higher ethanol concentrations than th starting strain at 40°C, respectively.	(Lin et al. 2019)
	Increase the global mutation rate by overexpressing mutated genes in the genome or mutated genes on mutagenic plasmid vectors (including MP6, TaGTEAM, MutaT7, EvolvR, PACE, Retrons, etc.) such as Genome Replication Engineering Assisted Continuous Evolution (GREACE)	The use of eMutaT7 increased the minimum inhibitory concentration of the antibiotic cefotaxime for the strai from 0.06 to 800 mg/L. The minimum inhibitory concentration for ceftazidime was increased from 0.2 mg/L to 4 g/L.	(Park & Kim 2021)
	Increasing global mutation rates by disruption of the native DNA mismatch repair system, introducing mutant DNA polymerases or heterogenous R/M system.	A mutant library was constructed by deleting the *mutS* gene encoding the DNA mismatch repair enzyme in *L. casei*, and an evolved strain that could grow well at pH 2.5 with a 10-fold higher survival rate was obtained.	(Overbeck et al. 2017)
Genetic diversification by introducing in vitro generated targeted DNA variants	Global Transcriptional Mechanism Engineering (gTME) utilizes an error-prone plasmid library of global cellular transcriptional machinery to induce global perturbations of the transcriptome and unlock complex phenotypes.	A mutant library of the *rpoA* was constructed in *E. coli* by gTME, and a total of 409 mutants were obtained. The mutant strain BTR1:BRM-353 was able to produce 104.3 mg/L of indigo, representing a 204% increase.	(Alper & Stephanopoulos 2007)
	Obtain mutated DNA from customized synthetic oligo pool, when used as integration donor fragments, multiplex automated genome engineering (MAGE) and CRISPR-enabled trackable genome engineering (CREATE) are created and applied for genome-wide mapping of mutations at single or multi-nucleotide resolution.	The mutant Crp S28P, which was able to tolerate 2 g/L furfural, was obtained by combining CREATE with ALE. The biomass of the evolved strain increased by 50% compared with the wild type after 8 h of growth.	(Garst et al. 2017)

selection' as the core principle, and successfully isolated *E. coli* strains capable of growing in the presence of 1.25% n-butanol, and increased the growth rate of the strain in the presence of 0.1% acetic acid eight-fold compared to the initial strain (Luan et al. 2013). These results proved that GREACE can accelerate the process of evolution under certain conditions. Choe *et al.* used ALE to obtain the *mutS* gene deleted strain of *E. coli* eMS57 and the *mutS*-restored strain eMS57mutS$^+$. The eMS57 strain accumulated a total of 102 new mutations in two replicate cultures, whereas the eMS57mutS$^+$ strain contained only 26 mutations Thus, the deletion of *mutS* increased the mutation rate and allowed the strain to adapt more quickly to specific conditions (Choe et al. 2019).

The R-M system is a prokaryotic innate immune system (Stern & Sorek 2011), including two enzymes that play the roles of recognition and protection (Tock & Dryden, 2005; Labrie et al. 2010). Yoko *et al.* introduced an additional exogenous R-M system derived from the *Pseudomonas aeruginosa* R-M system into the host *E. coli* YA027, which caused incomplete methylation of the nascent host genome through unbalanced distribution or unbalanced expression of restriction endonuclease and methylation enzymes at each cell division. As a result, restriction endonuclease shears the genome and subsequently generates a series of insertions/duplications (Yoko et al. 2011). Continuous evolution over 172 days resulted in a seven-fold increase of the initial growth rate. Furthermore, the r$^+$m$^+$ strain was better than the r$^-$m$^+$ strain in both growth rate and the insertion sequence (IS) polymorphism genotype, suggesting that the evolution rate of the r$^+$m$^+$ strain is faster than that of the r$^-$m$^+$ strain. The results of this study provide a new research idea for evolutionary engineering by regulating the R-M system to continuously enhance the natural mutation rate of the strain. Bai *et al.* designed an R-M system-mediated genome editing (RMGE) technique in *E. coli*. The concentration of cadaverine produced by the evolved strain EC3053 after ALE was 3.35 ± 0.14 g/L (Bai et al. 2018). Garst *et al.* used CREATE to study the evolution of thermotolerance in *E. coli* (Garst et al. 2017), which not only efficiently tested the results of a year-long ALE study, but also simultaneously identified an additional 141 mutants (a 61% increase). In addition, they also obtained a Crp S28P mutant, which can tolerate 2.0 g/L furfural. After 8 h of growth, the biomass accumulation of Crp S28P increased by 50% compared with the wild type. In addition to the above method, global transcription machinery engineering (gTME) changes key regulatory proteins by error-prone PCR or DNA shuffling, thereby forming a new type of diversity at the transcriptional level (Liu et al. 2018). This system is also a powerful tool for constructing mutant libraries (Li et al. 2018; Xue et al. 2019; Deng et al. 2021). Alper and Stephanopoulos constructed 10^6 mutants of *rpoD*, and performed gTME studies, which finally increased the tolerance of *E. coli* to ethanol to 70 g/L (Alper & Stephanopoulos 2007). In order to improve the yield of indigo, Chen *et al.* constructed a mutant library of the *rpoA* gene of *E. coli* by gTME, and obtained 409 mutants in total (Chen et al. 2021). The mutant strain BTR1: BRM-353 obtained by screening and isolation was able to produce 104.3 mg/L of indigo, an increase of 204%. Du *et al.* used gTME to build a mutant library of the *SPT15* gene of *S. cerevisiae* and screened more than 1 000 strains for reduced ethanol production (Du et al. 2020). The best mutant strain produced 35.33 ± 3.09 g/L of ethanol, which was 34.9% lower than that of the reference strain. In addition, the ethanol yield decreased from 0.36 ± 0.01 to 0.24 ± 0.02 g/g sugar. El-Rotail *et al.* constructed 150 mutants of the *SPT15* and *TAF23* genes of *S. cerevisiae* using gTME and verified their ethanol tolerance and yield (El-Rotail et al. 2017). The ethanol tolerance level of the mutants was improved up to 10% (v/v), and the cell density reached 2.07 g/L (DCW), compared to 1.99 g/L (DCW) in the control strain. In addition, the highest ethanol yield of the mutant reached 15.72 g/L, which was 60.4% higher than that of the control strain.

Reverse engineering and genome editing for resolving evolutionary mechanisms

Due to the random nature of mutations, the evolved strains accumulate beneficial as well as neutral and deleterious mutations. Unfortunately, the beneficial mutations usually account for a small proportion, resulting in an unclear genetic background of the evolved strains, which poses a problem for the elucidation of the evolutionary mechanism (Kawai et al. 2019; Yuan et al. 2019). With the development of omics technologies, such as genomics, transcriptomics, and metabolomics, reverse engineering can be applied to effectively identify beneficial mutations, elucidate evolutionary mechanisms, and further reconstitute engineered strains carrying only beneficial mutations (Warner et al. 2009; Peano et al. 2014). Such reconstituted strains have the advantages of stable genetic characteristics and a clear genetic background (Fig. 2).

Prell *et al.* improved the ability of *C. glutamicum* to produce glutarate by adaptive evolution and isolated faster growing strains for reverse genetic engineering studies by serial dilution growth experiments (Prell et al. 2021). The results showed that the deletion of the *gdh* gene leads to flux enforcement and thus the exchange of amino acids in the large subunit of L-glutamate acid-2-hydroxyglutarate aminotransferase, which in turn accelerates the production of glutarate. Chen *et al.* reverse-engineered *E. coli* for increased octanoic acid (C8) production based on mutations identified through adaptive evolution. It was found that both *waaG* and *rpoC* mutations increase the concentration of C8, whereby *rpoC* mutation appears to be the main driver of this effect. In addition to the above two genes, mutations affecting the BasS-BasR two component system can also increase the growth rate and cell surface hydrophobicity (Chen et al. 2020). Perli *et al.* enabled *S. cerevisiae* to grow normally in the absence of pyridoxine by ALE. After reverse engineering, it was found that the mutated transcription activator *Bas1* in the strain is involved in the regulation of purine and histidine, and also reduces the inhibition of *SNZ1*-related genes, thereby increasing the biosynthesis of pyridoxine and enabling the strain to grow normally (Perli et al. 2020). Yayo *et al.* performed laboratory evolution of *C. thermocellum* with increasing fructose concentrations and decreasing cellobiose concentrations to explore its growth on fructose and underlying molecular mechanisms, followed by reverse metabolic engineering based on comparative genome sequencing (Yayo et al. 2021). It was found that biomass yields on fructose increased from 0.15 ± 0.01 to 0.18 ± 0.01 g/g in the evolved strain, while the deletion of the transcriptional regulator gene Clo1313_1 831 increased fructokinase activity and shortened the lag time on fructose. The additional introduction of a G-to-V mutation at position 148 of *cbpA* allowed the strain to grow immediately on fructose. Gassler *et al.* increased the growth rate of *Pichia pastoris* to 0.018h^{-1} by ALE method and analyzed the autotrophic phenotype of *P. pastoris* by reverse engineering (Gassler et al. 2022). The results showed that the activities of mutant enzymes Nma1 and Prk affected the growth rate of the strain, and lower enzyme activities were more conducive to the expression of the strain's autotrophic phenotype.

Wang *et al.* improved the tolerance of *Y. lipolytica* to ferulic acid by ALE, and the evolved strain was tolerant to 1.5 g/L of ferulic

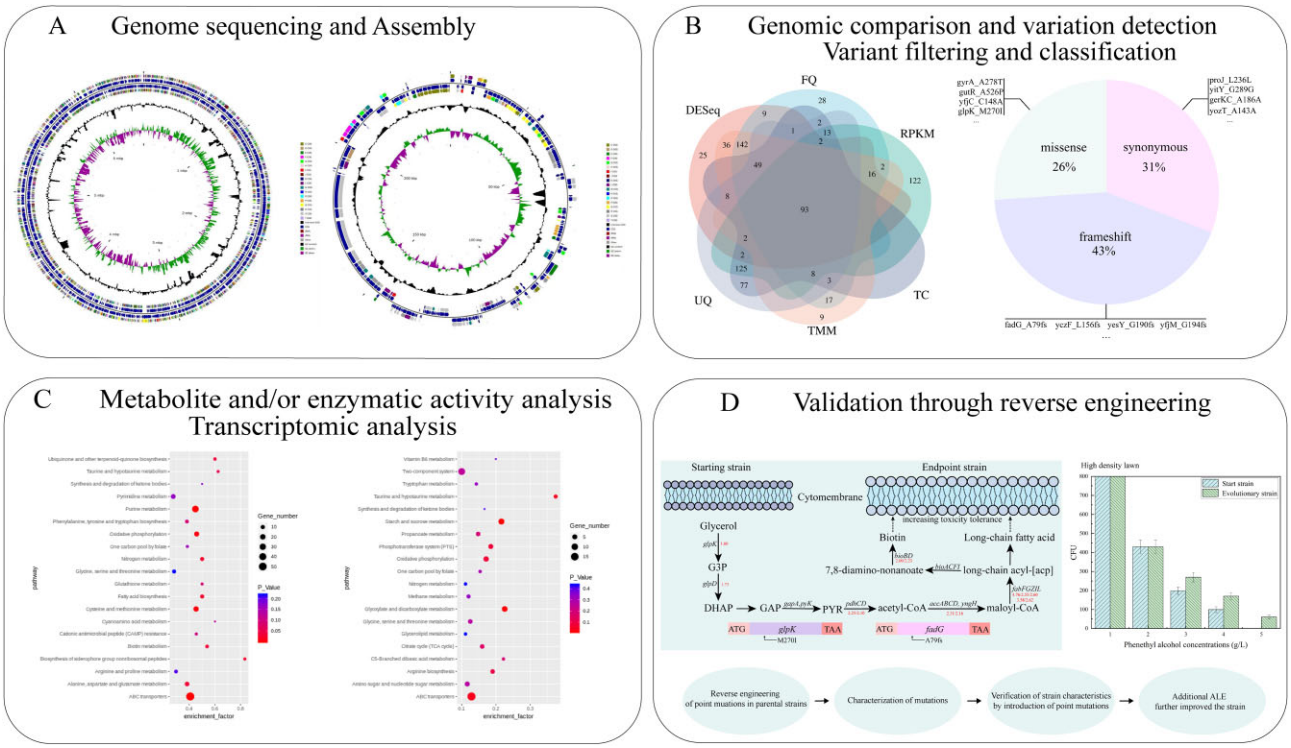

Fig. 2 Analysis of gene mutations in the evolved strains with unclear genetic background by multi-omics technology combined with reverse metabolic engineering. A: The whole genome of the evolved strain is sequenced and assembled; B: The evolved gene mutations are classified by comparative genomics to detect variants; C: Metabolite, enzymology and transcriptome analysis are performed on the evolved strains to analyze the genetic basis of the target phenotype; D: Reintroducing specific evolved mutations into wild-type strain by reverse metabolic engineering restores the evolved phenotype and confirms the validity of identified beneficial mutations, resulting in improved strains with a well-defined genetic background.

acid (Wang et al. 2021). They used transcriptome analysis and reverse engineering to verify that YALI0_E25201g, YALI0_F05984g, YALI0_B18854g, and YALI0_F16731g were among the most highly upregulated genes. Individual overexpression of the four genes could also enhance the tolerance of ferulic acid. Liu *et al.* combined metabolic engineering and adaptive evolution to increase the growth rate and PA production capacity of *P. acidipropionici* (Liu et al. 2020). The thus obtained mutant strains were analyzed by transcriptomics and proteomics, which revealed that the differentially expressed genes (EDGs) and proteins (DEPs) in the strains were involved in glycolysis and tricarboxylic acid cycle, thus affecting the energy generation capacity of the cell. In addition, the above genes were all upregulated, providing more energy and precursors for the synthesis of PA. Wang *et al.* further investigated the tolerance of *E. coli* to branched-chain higher alcohols (BCHAs) by means of transcriptomic and metabolic modifications (Wang et al. 2020). Gene mutations of the global stress response regulator RpoS (*rssB*, *acrB*, *clpX*) enhanced energy metabolism through three pathways, which was crucial for increasing the tolerance of *E. coli* to BCHAs. Furthermore, they also proposed multiple genetic engineering targets for increasing the tolerance of *E. coli*. Henson *et al.* increased the substrate utilization rate and tolerance of *R. opacus* PVHG6 to aromatic mixtures by increasing the concentration of aromatic compounds in the medium. Further transcriptome analysis revealed that five genes associated with ABC transporters or ABC transporter-related proteins were upregulated, suggesting that ABC transporters may be involved in the uptake or efflux of aromatic compounds. In addition, two genes related to acetyl-CoA acetyltransferases were also upregulated, which may increase the metabolic flux through the ketoadipic acid pathway. This study confirms the significance of combining ALE with multi-omics methods (Henson et al. 2018). As a non-model species, the cooperative utilization model of *R. opacus* is important for the development of predictive metabolic models. Roell *et al.* developed a genome-scale metabolic model that can predict metabolic fluxes of cells grown on different carbon sources. Interestingly, they argued that genetic differences induced by adaptive evolution do not lead to changes in the flux network. They also subjected *R. opacus* to comprehensive omics analysis, which established the relationship among metabolome, transcriptome, and fluxome, enabling a deeper mechanistic understanding of the aromatic utilization mechanism (Roell et al. 2019).

Wang *et al.* systematically analyzed the mutations and transcriptional expression pattern of mutant genome-shuffled riboflavin overproduction strain *B. subtilis* 24/pMX45 by whole-genome sequencing and transcriptomic analysis combined with reverse metabolic engineering. Several beneficial mutations associated with riboflavin overaccumulation, such as RibC (G199D), *ribD*+ (G + 39A), PurA (P242L), CcpN (A44S), YvrH (R222Q), YhcF (R90*) and YwaA (Q68*) were screened by reverse metabolic engineering and markerless allele replacement techniques (Wang et al. 2018). Su *et al.* screened *S. cerevisiae* with high isobutanol tolerance by EMS mutagenesis followed by ALE, and further increased the isobutanol titer through metabolic engineering (Su et al. 2021). Whole-genome sequencing showed that a total of 59 genes were mutated, and these genes were mainly enriched in cell growth, basal transcription factors, oxidative stress response, and related pathways. In addition, they found that overexpression of *CWP2* and *SRP40*[39] could improve the tolerance of *S. cerevisiae* to isobutanol through reverse engineering experiments. Choe *et al.*

obtained the evolved strain eMS57 from *E. coli* MS56 with 55 deletions and gradually decreased the LB content for 807 generations. The growth rate and cell density of the evolved strain reached the same level as that of the strain without gene deletion. The metabolic rewiring was found to be globally orchestrated by mutations in *rpoD*, which altered the promoter binding pattern of RNA polymerase. Further multi-omics analyses revealed that remodeling of the transcriptome and proteome of eMS57 reoptimized the metabolism to recover normal growth (Choe et al. 2019). Mohamed *et al.* improved the tolerance of *Pseudomonas putida* KT2440 to p-coumaric acid (pCA) and ferulic acid (FA) by tolerance ALE (TALE) (Mohamed et al. 2020). Whole-genome resequencing revealed genetic targets associated with the degradation of aromatics and enhanced toxicity tolerance, with *PP_3* 350 being the most frequently mutated gene. This is because pCA/FA acts in the periplasm and/or cytoplasm, and the mutation of *PP_3* 350 restricts their entry, thereby reducing toxicity. Srivastava *et al.* improved the tolerance of *Synechococcus elongatus* to n-butanol by ALE (Srivastava et al. 2021). Whole genome sequencing of n-butanol-adapted strains revealed mutations in RpoB and ABC transporters. Among them, *rpoB* was directly involved in the tolerance to n-butanol, indicating that the amino acid substitution mutation of RpoB enhanced the tolerance of the strain. In addition, ferrous ions can react to release harmful free radicals under oxidative stress caused by n-butanol, while ABC transporters control the intracellular level of ferrous ions to improve strain tolerance. Lou *et al.* newly developed a high-throughput plate-based screening method to identify specific SNPs contributing to the high-temperature and high-light tolerance of Sye2973. They found that the C252Y mutation in the *atpA* gene (encoding the α subunit of F_oF_1 ATP synthase) plays a major role in the stress tolerance of *Synechococcus elongatus* UTEX 2973. Additional experiments showed that mutations can increase both protein levels and intracellular activity of F_oF_1 ATP synthase, thereby improving the tolerance of the strain (Lou et al. 2018). Ungerer *et al.* used comparative genomics to identify three genes that allow *S. elongatus* 2 973 to grow rapidly. Among them, the fast-growth-related allele of *atpA* produces an ATP synthase with higher specific activity, and *ppnK* encodes a NAD^+ kinase that can significantly improve the kinetics. Overall, four SNPs in three proteins play a decisive role in the rapid growth of *Synechococcus* (Ungerer et al. 2018). However, Zhou and Li cultured and sequenced *S. elongatus* 2 973 and *S. elongatus* 7 942 under the same high-light conditions. The results showed that *S. elongatus* 7 942 was mutated and grew as fast as *S. elongatus* 2 973, but no SNPs were found in the *atpA* gene of *S. elongatus* 7942. Therefore, the authors concluded that the SNPs responsible for stress tolerance may be variable, and the relationship between SNPs and growth rate needs further study (Zhou & Li 2019).

Concluding remarks and future perspectives

ALE is a highly effective means for obtaining target strains with specific phenotypes that make them more productive or tolerant to adverse environments relevant to industrial applications. Adaptive evolution has a wide range of applications in microbial breeding for metabolic pathway activation, substrate utilization, optimization of microbial growth phenotypes, and overaccumulation of target metabolites. Due to the development of massively parallel microbial culture techniques, high-throughput sequencing, bioinformatics, and genome editing technologies, adaptive evolution has become a powerful breeding tool, which also provides an important research basis for genomic analysis, mechanistic studies, and rational metabolic engineering. However, ALE technology still has many problems to overcome. For example, the evolutionary process is often time-consuming and labor-intensive, which is an important reason limiting the widespread use of this technology. ALE technology should be closely integrated with rational metabolic engineering to maximize its role in rapidly improving strain phenotypes. Compared to rational metabolic engineering, which introduces exogenous genes directly or knocks out the original genes, ALE cannot significantly reshape metabolism in a short period of time, but if a rationally engineered strain is used as the initial strain for evolution, it can significantly shorten the evolutionary process, especially for the acquisition of complex phenotypes, where the combination of metabolic engineering and ALE is more effective. In addition, the genetic stability of the constructed industrial strains is also an important issue, which will cause serious losses in the process of fermentation scale-up. Fully understanding ALE and combining it with systems biology approaches can provide insights into strain stability, leading to a reduction of deleterious mutations and an increase of production stability, facilitating the transfer of the strain to industrial-scale applications. Notably, the growth-coupled ALE design is an excellent choice to address this issue (Czajka et al. 2017; Czajka et al. 2020).

Furthermore, although the accumulation of mutations during evolution can be analyzed by technologies such as genome and transcriptome sequencing, the random nature of mutations results in a complex and often intractable evolutionary mechanism, which makes it difficult and time-consuming to identify the relevant beneficial mutations for reverse engineering. With the widespread use of ALE, mutation analysis and elucidation of evolutionary mechanisms has become increasingly important, and the investigation of genotype-phenotype relationships has become an important area of research. Although reverse metabolic engineering has made great progress, its scope still limited, and the interpretation of genotype-phenotype associations in ALE studies still lacks standardization. To solve this problem, more multi-omics techniques should be used in future studies to fully integrate genomic, transcriptomic, proteomic, metabolomics, and fluxomic information and thereby increase the available data for the identification of phenotype-genotype associations at the systems biology level. ALE technology has been used to systematically resolve the evolutionary mechanisms at the levels of microbial metabolism and gene regulation (Henson et al. 2018). In conclusion, ALE technology facilitates the selection of industrial production strains, and the development of related technologies has led to its rapid application as an important tool for industrial microbial breeding strategies.

Author contributions

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The authors declare that they have no conflicts of interest.

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