Accelerating adaptive laboratory evolution via engineering of mutagenesis system for synthetic biology applications

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Abstract

Synthetic biology focuses on engineering microorganisms for applications in industrial production and disease treatment. However, commonly used model strains often lack adaptability to specific environmental or application contexts. Adaptive laboratory evolution offers a broad-spectrum strategy that exposes strains to simulated environmental pressures to gradually enhance their tolerance, but its widespread usage is limited by low efficiency. We present MP6-UTT, a genetic tool for stress-directed evolution that enables highly efficient, broad-spectrum mutagenesis within bacterial cells, significantly accelerating the evolutionary process. Using MP6-UTT, we enhanced *E. coli* BL21 (DE3)'s ability to utilize xylose and tolerate high salinity in seawater. We also improved the survival efficiency of *E. coli* Nissle 1917 under simulated intestinal conditions with tagatose as an additional carbon source, aiding probiotic colonization. Our platform offers a versatile and powerful approach for rapid microbial adaptation, facilitating the real-world translation of synthetic biology projects.

Keywords: Synthetic biology, Chassis, Adaptive laboratory evolution, Metabolic engineering, Therapeutic

1. Introduction

Many synthetic-biology–designed microbial chassis struggle to maintain performance under real-world industrial conditions. When scaled-up operations or process optimizations introduce fluctuations in pH, solvent levels, osmolarity, temperature, or nutrient availability, these engineered strains often exhibit reduced titers, metabolic instability, or require costly and precise process control to function reliably. To improve both usability and resilience, integrating stress evolution as a parallel engineering strategy is increasingly valuable [1]. This approach enhances the robustness of microbial hosts, making them better suited to tolerate industrial variability without constant human intervention or expensive safeguards.

Applying stress evolution before deployment typically delivers three key advantages: it broadens the operational tolerance range, keeping activity and yield more stable under perturbations; it increases strain robustness, reducing reliance on tight process constraints and minimizing scale-up risks; and it offers transferable adaptability, where gains under one stress condition often benefit related ones, thus accelerating optimization from lab to production. Nature achieves this through a cycle of pressure, selection, inheritance, and amplification. In the lab, this can be condensed by rapidly generating genetic diversity and applying targeted stress conditions. MP6 [2]—a controllable in vivo mutagenesis system—facilitates this by producing diverse substitution mutations (A/T and G/C) on demand. Its inducible design allows tight temporal control in a mutate–stress–select cycle, enabling populations to efficiently explore mutational space under specific pressures. Researchers can tailor composite or gradient stress schemes (e.g., salt, acid, solvent, or metal ion stress), then couple them with sequencing and phenotypic profiling to uncover adaptive mutation networks—effectively combining evolutionary pressure with directed genetic diversity.

In recent years, the rapid development of engineered base-modifying enzymes has created a new potential plug-and-play upgrade path for MP6. These enzymes include C→T and A→G editors [3-6] or any other basis for creating Apurinic/Apyrimidinic site (AP) sites with diverse sequence preferences expanded editing type and efficiency. The next-generation combination of base editors and MP6 could further accelerate stress-guided directed evolution for salt, acid, and solvent tolerance, as well as multifactorial adaptation—opening new frontiers for biomanufacturing and synthetic biology. Building on this concept, we developed a new generation of stress-evolution induction tools by integrating multiple base-modifying enzymes with MP6, and demonstrated their advantages in engineering *Escherichia coli* for improved adaptation to both industrial production conditions and simulated gut environments, offering new solutions for metabolic engineering and medical synthetic biology.

2. Result

2.1 Assessment of different base modifying enzymes in *Escherichia coli*

Several novel base editors have demonstrated high efficiency. TadA8e and TadA8r, both adenosine deaminases, catalyze adenine (A) deamination. During replication or repair, this deamination is recognized as guanine (G), enabling efficient A-to-G conversion. TDG3 efficiently excises thymine (T) in *E. coli*; the resulting abasic site is often repaired with adenine (A), showcasing its potential for creating base diversity. CDG4 excises cytosine (C), which is primarily replaced by adenine (A) or thymine (T), enabling multi-directional C editing. Together, these enzymes enrich the base-editing toolkit, enabling more flexible and efficient genetic modifications in prokaryotic and eukaryotic systems.

To test the mutagenic efficiency of these enzymes in *E. coli*, we used a reporter system based on a mutated chloramphenicol resistance gene (CmR). The start codon ATG was replaced with non-canonical codons (ATA, TTG, or CTG), disrupting translation initiation. Each variant served as substrates to test TadA8e, TadA8r, TDG3, and CDG4. Successful base editing that restored a functional start codon would confer chloramphenicol resistance, allowing growth on selective plates.

We synthesized genes for four base-modifying enzymes and expressed them using the constitutive plasmid plac. To validate plac's expression strength, we introduced the reporter *tsPurple*; resulting purple *E. coli* colonies confirmed high-level gene expression.

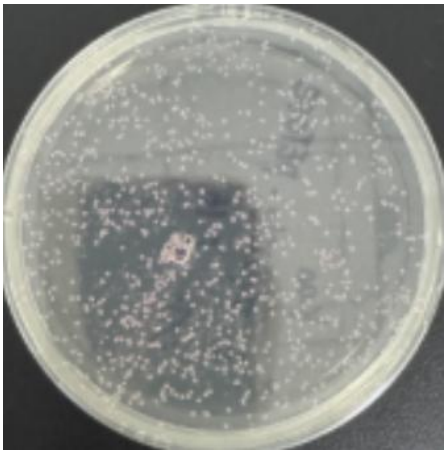


Fig.1 Expression of tsPurple under plac promoter.

The tsPurple gene was cloned under plac and transformed into DH5α, and selected in LB plate with spectinomycin under 37℃ for 12h.

However, when expressing the four enzymes from this vector, their cloning efficiencies varied significantly.

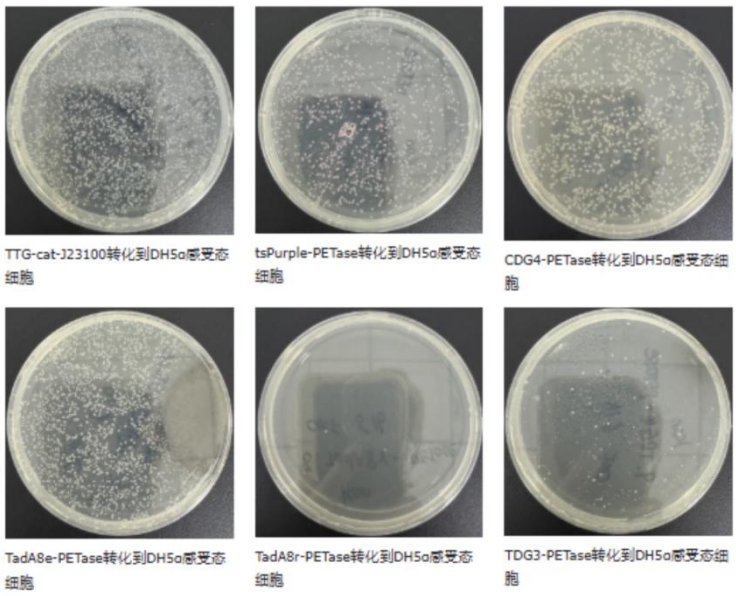


Fig.2 Construction of plac-CDG4, plac-TadA8e, plac-TadA8r and plac-TDG3.

We suspected the low construction efficiency was due to DNA-targeting enzyme cytotoxicity. To minimize toxicity for our mutagenesis system, we selected three less toxic enzymes—TadA8e, CDG4, and TDG3—for further testing.

We then introduced ATA-CmR with plac-TadA8e, CTG-CmR with plac-CDG4, and TTG-CmR with plac-TDG3 into *E. coli*. Transformants were cultured in LB medium and then plated on chloramphenicol-containing plates to assess editing efficiency (Figure XXX).

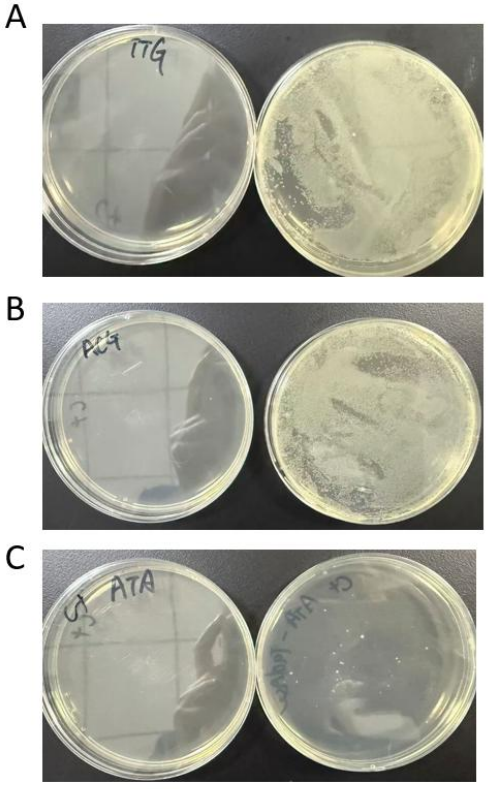


Fig.3 CmR rescue by three enzymes.

(A) TDG3, turning T to A, and rescue TTG to ATG start codon for CmR expression. (B) CDG4, turning C to T, and rescue ACG to ATG start codon for CmR expression (C) TadA8e, turning A to G, and rescue ATA to ATG start codon for CmR

expression. For each panel, E. coli transformed with CmR*-reporters (left) and reporters with enzyme-expressing plasmid (right) was cultured overnight and spread in chloramphenicol-containing LB plate.

2.2 Buildup of MP6-UTT

After confirming the mutagenic activities of TadA8e, CDG4, and TDG3, we aimed to incorporate them into the broad-spectrum mutagenesis plasmid MP6. Due to their cytotoxicity, expression required tight control using a low-leakage inducible promoter. We chose the arabinose-inducible pBAD promoter and verified its tight regulation using a pBAD-tsPurple construct. *tsPurple* expression was fully repressed without arabinose and strongly induced in its presence.

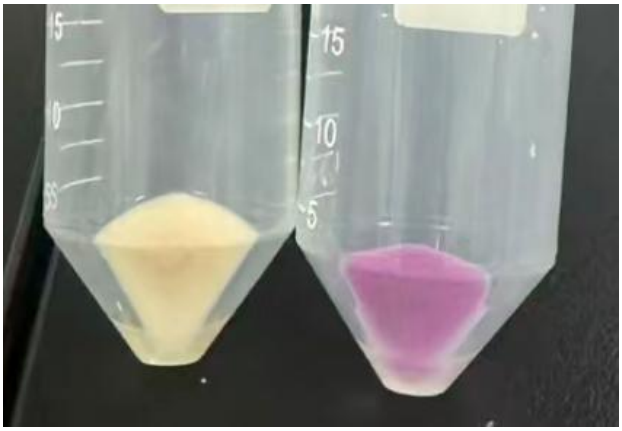


Fig.4 Performance of pBAD, as presented by tsPurple.

E. coli transformed with pBAD-tsPurple was cultured overnight without (left) or with 0.5% (m/v) L-arabinose (right).

Using the existing pBAD promoter in MP6, we inserted Pbad-TadA8e-CDG4-TDG3 between the plasmid's replication origin and resistance gene. This design ensures system stability: any recombination between the two pBAD promoters would result in loss of either the origin or resistance gene, preventing plasmid propagation. The correctly sequenced MP6-UTT plasmid was successfully constructed:

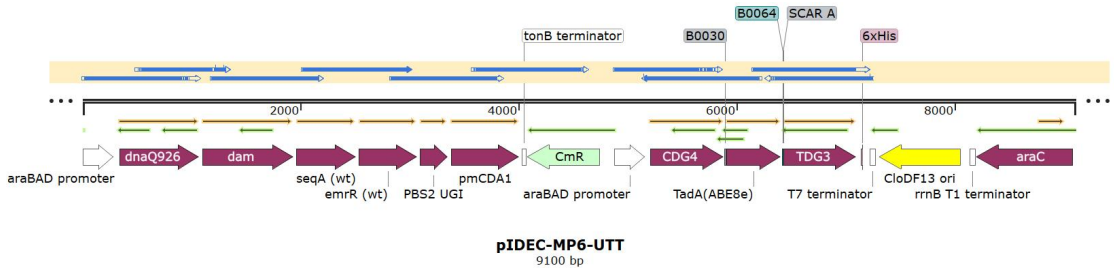


Fig.5 Sequencing result of MP6-UTT.

We introduced the construct into BL21, confirmed by colony PCR. Upon overnight induction with arabinose, no significant growth inhibition was observed, indicating the MP6-UTT system's suitability for stress-induced evolution in BL21. Similarly, in Nissle 1917, no growth inhibition was observed, suggesting its applicability there as well.

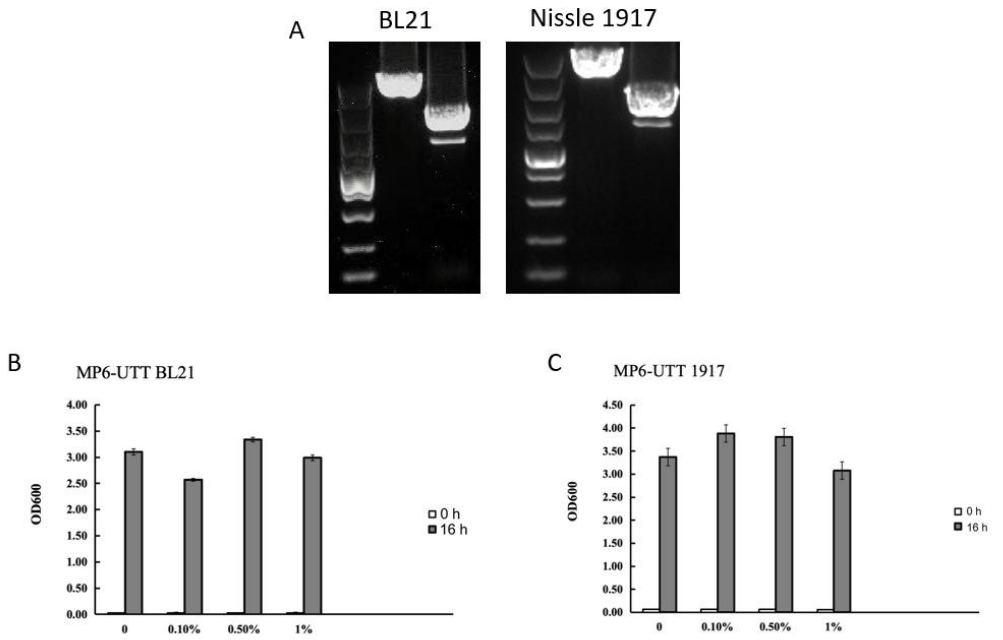


Fig.6 Test the compatibility of MP6-UTT with BL21 and Nissle 1917.

(A) Colony PCR results of BL21 (left) and Nissle 1917 (right) transformed with MP6-UTT. For each gel, maker, amplicon of the first gene cluster and of the second one were shown. (B) Growth of BL21-MP6-UTT without ot with MP6-UTT induction. (C) Growth of Nissle 1917-MP6-UTT without ot with MP6-UTT induction.

2.3 Stress-directed evolution of BL21(DE3) under industry-related stress

Engineering xylose utilization in *E. coli* BL21(DE3) offers key benefits for synthetic biology and industrial applications. As a major chassis for recombinant protein production, enhancing its ability to metabolize xylose allows growth on lignocellulosic hydrolysates–a cost-effective carbon source. This improves carbon efficiency, reduces glucose dependency, and supports the production of biofuels, biochemicals, and proteins. A xylose-optimized BL21(DE3) thus provides a more versatile and economical platform for research and biomanufacturing.

Application Area	Specific Products/Directions	Engineering Strategies
Biofuel Production	Ethanol, butanol, isopropanol; advanced fuels (fatty acid derivatives, alkanes) [1–2]	Enhance xylose metabolism to increase NADPH/acetyl-CoA supply
Bio-based Chemicals	Organic acids (lactic acid, succinic acid), amino acids (lysine, glutamate), sugar derivatives (xylitol, xylulose) [3–5]	Redirect metabolic flux toward target product pathways to improve yields
Lignocellulose Valorization	Mixed glucose + xylose fermentation for cost-efficient, high-yield biorefinery [6–7]	Eliminate carbon catabolite repression (CCR) to enable simultaneous glucose and xylose utilization

Protein Expression & Synthetic Integration	Using xylose as a carbon source for recombinant protein expression; coupling metabolism engineering with protein production in the same chassis [8–9]	Optimize chassis adaptability by integrating xylose metabolism with protein expression systems

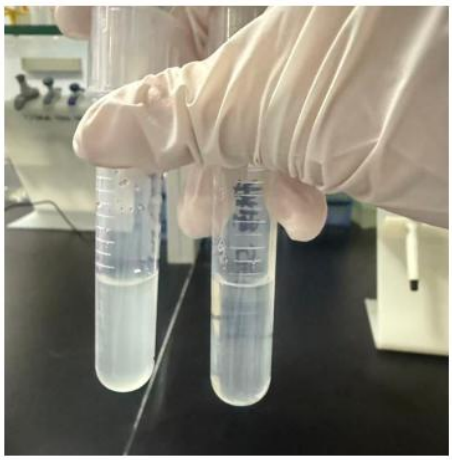
Adaptive laboratory evolution (ALE) under xylose stress effectively enhances xylose utilization in *E. coli* BL21. Unlike targeted metabolic engineering, this stress-driven approach enables the accumulation of beneficial mutations across global regulatory and metabolic networks, systemically optimizing transport, catabolism, and cofactor balance.

We improved xylose metabolism in BL21(DE3) using MP6-UTT by serial passage with arabinose induction (as in XX). Growth is shown in Figure XX. Evolved strains were streaked and compared to unevolved controls (Figure XX). Protein expression capability was confirmed by introducing plac-tsPurple; purple colonies after overnight culture indicated normal tsPurple production. These results demonstrate MP6-UTT rapidly enhances xylose metabolism in BL21(DE3) without compromising protein synthesis, suggesting industrial application potential.

A

		OD600	OD600	OD600
day1——MOPS+xylose overnight		0.518	0.514	0.516
day2,3——100 ul from day1, inoculate into 5ml, 1% L-Ara		0.784	0.785	0.784
day4——100 ul from day3, inoculate into 5ml, 1% L-Ara		0.739	0.738	0.733
day5——100 ul from day4, inoculate into 5ml, 1% L-Ara		0.683	0.688	0.687
day6——100 ul from day5, inoculate into 5ml, 1% L-Ara		0.66	0.666	0.661
day7——100 ul from day6, inoculate into 5ml, 1% L-Ara		0.958	0.96	0.964

B



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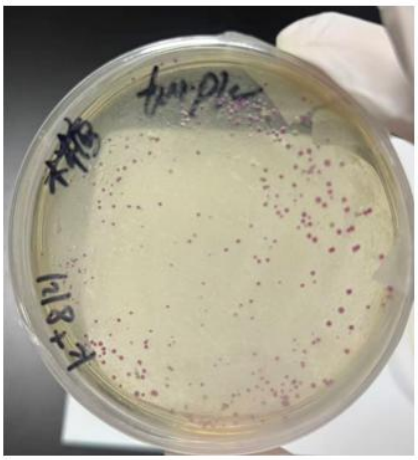


Fig.7 Improve growth of BL21(DE3) using xylose as carbon source by MP6-UTT.

(A) Daily passaging of BL21(DE3)-UTT in MOPS medium with xylose as the sole carbon source. The inoculation ratios and transfer conditions are shown on the left. A total of five passages were performed under identical conditions (37 °C, overnight). The bacterial growth after each overnight passage is shown on the right. (B) Comparison of growth between

the evolved strain and the wild-type strain (harboring only MP6-UTT without passaging) after completion of the adaptive evolution process. (C) Characterization of the protein synthesis capacity of the evolved strain, assessed using tsPurple, which causes colonies to appear purple.

2.4 Improve BL21(DE3) growth in simulate seawater

Seawater fermentation is superior to conventional freshwater-based methods in resource use, microbial adaptability, and energy efficiency. Using seawater as the medium conserves freshwater and provides a mineral-rich environment that supports specialized metabolism. High-salinity conditions favor halophilic or halotolerant strains, expanding the range of industrial hosts and enabling unique production pathways. The natural antimicrobial property of seawater also reduces sterilization needs, cutting energy consumption. This makes seawater fermentation a more sustainable and promising alternative for future industrial biotechnology.

2.5 Comparison: Traditional Fermentation vs. Seawater Fermentation

Aspect	Traditional Fermentation (Freshwater)	Seawater Fermentation
Water Source	Relies on large amounts of freshwater, requires additional salts	Uses natural or synthetic seawater, inherently contains multiple salts
Ionic Composition	Low-salt, mainly dependent on artificial formulation	High-salt (NaCl, Mg^{2+}, Ca^{2+}, etc.), mineral-rich environment
Microbial Hosts	Common hosts include E. coli, yeast, Bacillus subtilis, adapted to low-salt conditions	Requires halophilic/halotolerant strains or engineered conventional hosts
Osmotic Pressure	Low osmotic stress, minimal pressure on cells	High osmotic environment, cells must produce compatible solutes or adjust ion balance
Sterilization & Energy	Requires strict high-temperature, high-pressure sterilization → high energy consumption, strong dependence on steam/electricity	High-salt environment is naturally antimicrobial → lower sterilization needs, significantly reduced energy demand
Sustainability	Consumes freshwater, higher environmental pressure	Conserves freshwater, reduces energy dependence, aligned with green bioprocessing

Seawater tolerance engineering significantly broadens the application scope of *E. coli* BL21(DE3). This adaptation enables recombinant protein and metabolite production directly in seawater, reducing freshwater use and costs-especially beneficial in water-scarce regions. The salt-tolerant strain can utilize high-salinity substrates like marine biomass hydrolysates without

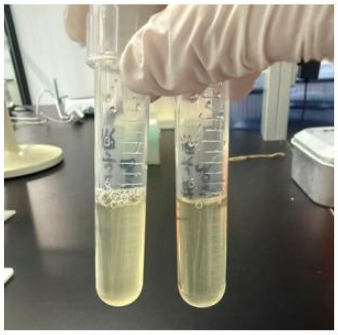
desalination. Furthermore, it serves as a chassis for marine applications such as oil spill bioremediation and plastic degradation, highlighting its dual potential for industrial biomanufacturing and environmental sustainability.

Using MP6-UTT, we optimized BL21(DE3) growth in simulated seawater by serial passaging under arabinose induction (as in XX). Growth profiles are shown in Figure XX. Evolved strains were streaked next to the parent (Figure XX), and protein expression was confirmed by introducing plac-tsPurple; purple colonies after overnight culture indicated normal tsPurple production. These results show that MP6-UTT rapidly improves seawater tolerance in BL21(DE3) without affecting protein synthesis, underscoring its industrial potential.

A

	OD600	OD600	OD600
day1- 1:9 (sea water : clear water)	0.909	0.906	0.906
day2- 3:7 (sea water : clear water) 100 ul from day1, inoculate into 5ml, 1% L-Ara	0.845	0.844	0.847
day3- 5:5 (sea water : clear water) 100 ul from day2, inoculate into 5ml, 1% L-Ara	0.382	0.378	0.379
day4- 5:5 (sea water : clear water) 100 ul from day3, inoculate into 5ml, 1% L-Ara	0.859	0.848	0.856
day5- 5:5 (sea water : clear water) 100 ul from day4, inoculate into 5ml, 1% L-Ara	0.628	0.629	0.629
day6- 6:4 (sea water : clear water) 100 ul from day5, inoculate into 5ml, 1% L-Ara	0.636	0.633	0.635
day7- 7:3 (sea water : clear water) 100 ul from day6, inoculate into 5ml, 1% L-Ara	0.449	0.451	0.451
day8- 8:2 (sea water : clear water) 100 ul from day7, inoculate into 5ml, 1% L-Ara	0.792	0.79	0.795
day9- 9:1 (sea water : clear water) 100 ul from day8, inoculate into 5ml, 1% L-Ara	0.411	0.413	0.413

B



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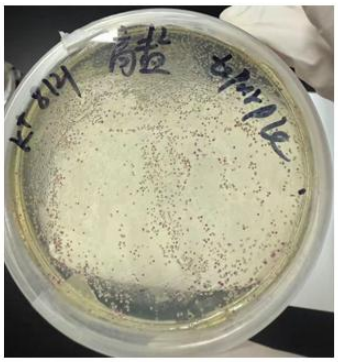

Fig.8 Improve growth of BL21(DE3) in seawater environment by MP6-UTT.

(A) Daily passaging of BL21(DE3)-UTT in mixed LB medium, prepared by combining LB made with simulated seawater and LB made with regular water. The specific inoculation ratios and transfer conditions for each passage are shown on the left. In total, eight passages were carried out under identical conditions (37 °C, overnight). The bacterial growth after each overnight passage is shown on the right. (B) After the adaptive evolution process, the evolved strain was compared with the wild-type strain (harboring only MP6-UTT without passaging) in LB medium prepared entirely with simulated seawater. (C) Characterization of the protein synthesis capacity of the evolved strain, assessed using tsPurple, which causes colonies to appear purple.

2.6 Stress-directed evolution of Nissle 1917 for gut-related applications

Synthetic biology offers a novel approach to treating intestinal diseases beyond traditional drugs or probiotics. Engineered bacteria can continuously produce therapeutics in the gut, dynamically sense environmental cues, and perform complex tasks like modulating metabolites, outcompeting pathogens, and regulating immunity. This combination of durability and precision makes them promising candidates for treating inflammatory bowel disease, infections, and metabolic syndromes.

A key challenge in using engineered bacteria to treat gut diseases is achieving stable colonization.

The intestinal environment is highly competitive, with resident microbiota monopolizing resources and adhesion sites. Even when therapeutic functions are added, engineered strains often fail to withstand microbial competition, immune clearance, and metabolic pressures. Consequently, their populations decline over time, limiting long-term persistence and therapeutic efficacy.

Enhancing engineered bacteria's efficiency in utilizing specific carbon sources can provide a nutritional advantage. By enabling them to metabolize underutilized dietary substrates, they can occupy a "dedicated ecological niche", promoting early colonization and strengthening therapeutic effect. While insufficient alone, combining this with adhesion enhancement and immune evasion can significantly improve their gut survival and stability.

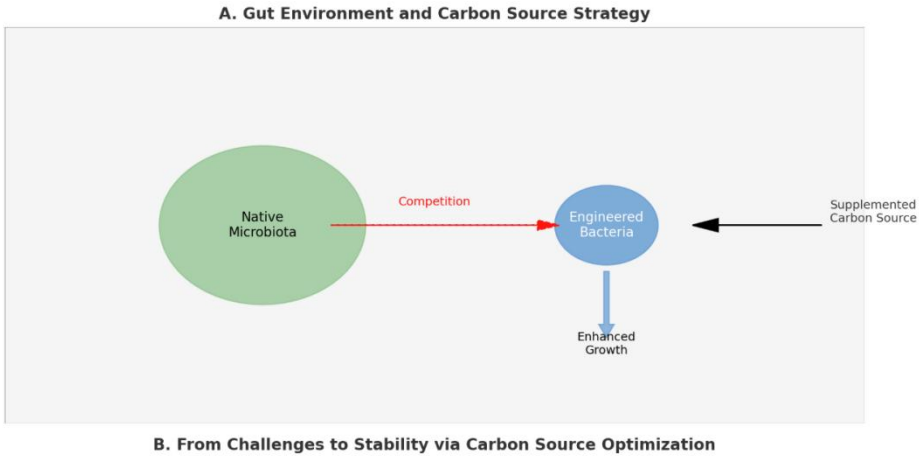

Fig.9 Challenges and potential solutions of gut probiotic therapy.

(A) Engineered probiotics may be displaced by wild-type microbial communities, leading to the loss of their ecological niche. This effect can be mitigated by supplementing a carbon source that only the engineered strain is able to utilize. (B) Schematic logic of how a specific carbon source compensates for the ecological niche.

We next applied MP6-UTT to improve tagatose utilization in *Escherichia coli* Nissle 1917. Tagatose, a food-grade sugar metabolized by few microbes, serves as a relatively specific carbon source for this strain.

As described in XX, the MP6-UTT plasmid was serially passaged with continuous arabinose induction. Growth after each passage is shown in Figure XX. After evolution, the strains were streaked and compared to the parent in monichangye medium (Figure XX), confirming that MP6-UTT rapidly generated Nissle 1917 variants with enhanced tagatose utilization.

Furthermore, this approach lays the foundation for continued adaptive evolution or for metabolic engineering toward alternative carbon sources, thereby enhancing the therapeutic potential of Nissle 1917 in treating intestinal disorders.

A

		OD600	OD600	OD600
day1 - MOPS+tagatose, overnight		0.712	0.709	0.712
day2 - MOPS+tagatose, overnight 100 ul from day1 inoculated into 5ml, 1% L-Ara		0.605	0.608	0.607
day3 - MOPS+tagatose, overnight 100 ul from day2 inoculated into 5ml, 1% L-Ara		0.54	0.539	0.542
day4 - MOPS+tagatose, overnight 100 ul from day3 inoculated into 5ml, 1% L-Ara		0.574	0.577	0.574
day5 - MOPS+tagatose, overnight 100 ul from day4 inoculated into 5ml, 1% L-Ara		0.483	0.483	0.485
day6 - MOPS+tagatose, overnight 100 ul from day5 inoculated into 5ml, 1% L-Ara		0.524	0.526	0.528
day7 - MOPS+tagatose, overnight 100 ul from day6 inoculated into 5ml, 1% L-Ara		0.568	0.565	0.566

B

C

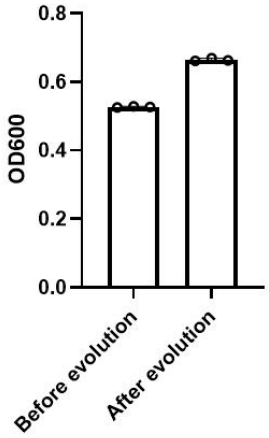

Fig.10 Improve growth of Nissle 1917 in gut-stimulated environment with tagatose by MP6-UTT.

(A) Daily passaging of Nissle 1917-MP6-UTT in MOPS medium with tagatose. In total, eight passages were carried out under identical conditions (37 °C, overnight). The bacterial growth after each overnight passage is shown on the right. (B) After the adaptive evolution process, the evolved strain was compared with the wild-type strain (harboring only MP6-UTT without passaging) in simulate intestinal fluid with tagatose. (C) Statistical analysis of the growth performance of the strains before and after evolution.

3. Discussion

Synthetic biology focuses on engineering microbial strains for industrial or therapeutic applications. Although numerous strategies exist, most have been implemented in well-established model strains like *E. coli* DH5α, MG1655, and Nissle 1917. Consequently, the resulting strains may not be optimally suited for specific application scenarios, limiting their practical utility. Directed evolution—particularly stress-based evolution addresses this by gradually applying selective pressure to mimic real-world conditions, thereby improving their strain tolerance and applicability. Traditionally, stress evolution has been performed through serial passaging or accelerated by introducing broad-spectrum mutagenesis tools such as MP6. However, as the field evolves, more rapid and efficient methods are urgently needed.

We developed the novel mutagenesis platform MP6-UTT by incorporating additional base-modifying enzymes into the existing MP6 system. The original MP6 plasmid contains enzymes that impair DNA repair pathways, disrupt base pairing, and induce cytosine deamination. Recent studies have expanded the toolkit of mutagenic enzymes, including adenine deaminases like TadA family and enzymes that create abasic (AP) sites by excising DNA bases, leading to base substitutions. We first screened several of these enzymes using an antibiotic resistance recovery assay and identified TadA8e, TDG3, and CDG4 as highly effective in introducing mutations in *E. coli*. Importantly, these enzymes, when combined with MP6, exhibited minimal toxicity to host cells, making them well-suited for stress-directed evolution.

We validated MP6-UTT using two strains: *E. coli* BL21 (DE3) and *E. coli* Nissle 1917. BL21 is widely used in protein expression and metabolic engineering. We demonstrated that MP6-UTT could enhance BL21's metabolic efficiency in utilizing carbon sources such as xylose, thereby expanding its applicability to bioconversion tasks such as agricultural waste (e.g., straw) processing—an important component of the carbon cycle. Furthermore, MP6-UTT enabled BL21 to develop salt tolerance within one week, enhancing its growth in seawater. This trait is highly desirable in next-generation microbial fermentation technologies, where seawater serves as a cost-effective and contamination-resistant medium. In parallel, we explored the use of MP6-UTT in *E. coli* Nissle 1917, a strain extensively used in synthetic biology for gut-related therapeutic applications. One of the major limitations of Nissle 1917 is its poor persistence in the gut environment due to rapid clearance, unless supplemented with additional carbon sources to enhance its ecological fitness. Using MP6-UTT, we improved Nissle's ability to metabolize tagatose. Within just one week, we observed a significant improvement in its performance in simulated intestinal fluid, indicating enhanced therapeutic efficacy.

MP6-UTT represent a powerful and versatile platform for stress-directed evolution and microbial strain engineering in synthetic biology. MP6-UTT is a broadly applicable system and can be extended to other microbial hosts beyond *E. coli*, like *Bacillus subtilis* or *Vibrio natriegens*. Furthermore, its use is not limited to laboratory strains or simple tasks; it holds promise for more complex applications such as space synthetic biology, context-specific biowaste recycling, and targeted cancer therapy. Collectively, MP6-UTT serves as an efficient and accessible evolution engine for rapidly developing robust microbial strains. We believe it will significantly accelerate both fundamental research and industrial translation in synthetic biology.

4. Materials and Methods

4.1 Plasmid construction

Plasmid construction was carried out using standard molecular biology techniques. All genes were synthesized by Tsingke, while plasmid backbones were obtained from previously prepared stocks. PCR amplification was performed using 2 × Phanta Flash Master Mix (Dye Plus), and gene

assembly was carried out with ClonExpress Ultra One Step Cloning Kit V2-C116, followed by synthesis confirmation at Tsingke. Plasmids were extracted using a plasmid extraction kit, and all procedures were performed strictly according to the manufacturer's instructions.

4.2 CmR recovery assay

The plac-TDG3, plac-CDG4, or plac-TadA8e plasmid, together with the corresponding Cm^R plasmid carrying a start codon defect, was co-transformed into E. coli DH5α. The transformation mixtures were plated on selective agar plates containing streptomycin and kanamycin. Successful transformants were subsequently cultured overnight in LB liquid medium, then spread onto chloramphenicol-containing agar plates. After overnight incubation at 37 °C, colony growth was examined.

4.3 Evolution by MP6-CTT

Induction of MP6-CTT was uniformly performed with 1% L-arabinose. For the different cases described in the manuscript, serial passaging was carried out according to the procedures illustrated in the corresponding figures. For the first generation, all cultures were initiated by overnight growth in either LB medium or MOPS minimal medium supplemented with the corresponding nutrient source, in order to obtain sufficient starter cultures.

4.4 Growth assay

Both the non-evolved and evolved strains were streaked on standard LB agar plates, and single colonies were inoculated into LB liquid medium for overnight culture. The overnight cultures were then centrifuged, and the supernatant was discarded. The cell pellets were resuspended in PBS, and this washing process was repeated three times to thoroughly remove residual LB medium. Subsequently, the cells were inoculated into media designed to simulate stress conditions, including MOPS–xylose medium, LB medium prepared with 100% artificial seawater, and a simulated intestinal fluid medium (a 1:1 mixture of MOPS minimal medium and simulated intestinal fluid). After overnight incubation, cultures were examined visually and/or measured for OD600.

4.5 Protein expression ability assay

The evolved strains were streaked onto LB agar plates, and single colonies were inoculated into LB liquid medium for overnight culture. Subsequently, chemically competent cells were prepared using the calcium chloride method, followed by transformation with the plac-tsPurple plasmid. The transformation products were plated onto kanamycin-containing LB agar plates and incubated overnight at 37 °C, after which the emergence of purple-colored colonies was examined.

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