Lambda RED Homologous Recombination-Mediated Knockout of thyA and dapA Genes in *Escherichia coli*

1. Introduction

Engineered *Escherichia coli* has emerged as a promising platform for targeted cancer therapy, owing to its capacity for heterologous gene expression, tumor tropism, and controllable biological activities. In this study, an engineered *E. coli* strain was constructed to produce cyclic diguanosine monophosphate (c-di-GMP)—a second messenger that not only activates the stimulator of interferon genes (STING) pathway in macrophages to polarize them toward an antitumor phenotype but also promotes bacterial biofilm formation. This biofilm formation enables the engineered bacteria to be retained within the tumor microenvironment (TME), enhancing local therapeutic efficacy. However, the potential for uncontrolled proliferation of engineered bacteria outside the TME poses a critical safety concern, necessitating the development of a strict "tumor-restricted survival" system.

Thymidylate synthase A (encoded by thyA) and dihydrodipicolinate synthase A (encoded by dapA) are essential genes for *E. coli* survival. The thyA gene is responsible for synthesizing thymidylate, a key precursor for DNA replication; its deletion renders bacteria auxotrophic for thymidine, meaning they can only survive in environments supplemented with exogenous thymidine. The dapA gene is involved in the biosynthesis of diaminopimelic acid (DAP), an indispensable component of the *E. coli* cell wall; deletion of dapA results in DAP auxotrophy, as DAP cannot be synthesized de novo and must be acquired from the surroundings. Notably, the TME is characterized by abnormal metabolism, with elevated levels of thymidine and DAP compared to normal tissues—an attribute that can support the growth of thyA^{-}/dapA^{-} double-knockout bacteria. In contrast, normal tissues lack sufficient concentrations of these nutrients, preventing the proliferation of the engineered strain outside the TME.

The λ-RED homologous recombination system is a powerful tool for precise gene editing in *E. coli*, consisting of three key proteins (Exo, Beta, and Gam) encoded by the exo, bet, and gam genes on the temperature-sensitive plasmid pKD46. Exo is a 5'→3' exonuclease that generates single-stranded DNA (ssDNA) overhangs from double-stranded DNA (dsDNA) targeting fragments; Beta is a ssDNA-binding protein that mediates annealing of these overhangs to homologous sequences in the *E. coli* genome; and Gam inhibits the host RecBCD nuclease, protecting the targeting fragment from degradation. This system enables efficient integration of antibiotic resistance cassettes (e.g., from plasmid pKD3) into the genome to replace target genes (thyA and dapA). Subsequent elimination of the resistance cassette and helper plasmids (pKD46 and pCP20) ensures the final engineered strain is marker-free, reducing potential interference with bacterial physiology and improving biosecurity.

This study aimed to use λ-RED homologous recombination to sequentially knockout the thyA and dapA genes in the c-di-GMP-producing *E. coli* strain. The resulting double-knockout strain was expected to exhibit strict auxotrophy for thymidine and DAP, limiting its survival exclusively to the TME while retaining the ability to produce c-di-GMP and form biofilms. This work lays the foundation for the safe application of engineered *E. coli* in targeted cancer immunotherapy.

2. Materials and Methods

2.1 Bacterial Strains and Plasmids

The host strain used in this study was *E. coli* designed to carry two plasmids: pUCP20-YedQ (expressing the YedQ protein to synthesize c-di-GMP) and a suicide plasmid derived from pUC18 (capable of AHL-induced lysis for c-di-GMP release, unrelated to biofilm formation). The helper plasmids included **pKD46** (temperature-sensitive, ampicillin-resistant, harboring the λ-RED recombination system under the control of the L-arabinose-inducible araB promoter), **pKD3** (containing a chloramphenicol resistance cassette flanked by FRT (FLP recognition target) sites, used as the template for amplifying targeting fragments), and **pCP20** (temperature-sensitive, ampicillin-resistant, expressing the FLP recombinase to excise FRT-flanked resistance cassettes).

2.2 Culture Media and Reagents

Luria-Bertani (LB) liquid medium and **LB agar plates** were prepared as standard; selective media were supplemented with **ampicillin (100 μg/mL), chloramphenicol (34 μg/mL), thymidine (5 mg/mL)**, or **DAP (8 mg/mL)** as required. A sterile **0.1g/mL working solution of L-arabinose** (for inducing λ-RED protein expression) was prepared in deionized water and filter-sterilized. Pre-chilled sterile reagents included **1×PBS buffer** (pH 7.4), **10% (v/v) glycerol**, and **10% (w/v) sucrose** (for electrocompetent cell preparation). PCR amplification was performed using **Vazyme 2×Phanta UniFi Master Mix-Dye Plus** (high-fidelity, for amplifying homologous arms and resistance cassettes) and **Vazyme 2×Taq Master Mix-Dye Plus** (for colony PCR verification). Other reagents included a **DNA gel extraction kit**, **plasmid mini-prep kit**, **agarose** (for gel electrophoresis), and **sterile ddH$_2$O**.

2.3 Primers

All primers were synthesized by a Sangon Biotech and resuspended in sterile ddH$_2$O to a final concentration of 10 μM. A comprehensive set of primers was designed for the construction of targeting fragments (with long and short homologous arms), verification of gene knockout events, and sequencing of genomic regions flanking the target loci (Table 1).

For thyA knockout with long homologous arms, a nested amplification strategy was employed using three pairs of primers to construct the targeting fragment:①thyA-F1 and thyA-R1 (with melting temperatures, T_m, of 60°C and 61°C, respectively) served as outer primers, incorporating extended homologous sequences (≥100 bp) complementary to the upstream and downstream regions of the thyA gene.②thyA-FRT-F (T_m=59°C) and thyA-FRT-R (T_m=60°C) acted as intermediate primers, overlapping with both the long homologous arms (amplified by thyA-F1/R1) and the FRT-flanked chloramphenicol resistance cassette (derived from pKD3), ensuring seamless fusion of the homologous arms with the resistance cassette.③thyA-F2 and thyA-R2 (both T_m=60°C) functioned as inner primers, refining the amplification of the final long-arm targeting fragment to ensure specificity and purity.

For thyA knockout with short homologous arms, a dedicated primer pair was designed:thyA short F (T_m=59°C) and thyA short R (optimized T_m=59°C) were used to amplify the targeting fragment containing shorter homologous sequences (30–50 bp) flanking the thyA gene, paired with annealing sites for the FRT-flanked resistance cassette. This pair enabled efficient construction of the short-arm targeting fragment, facilitating comparative analysis of recombination efficiency between long and short homologous arms.

For dapA knockout:①dapA-F1 and dapA-R1 (both T_m = 61°C) were used to amplify the targeting fragment, incorporating 100 bp homologous sequences flanking the dapA gene and annealing sites for the FRT-flanked resistance cassette.②FRT-2F (T_m = 59°C) and FRT-2R (T_m = 60°C) were employed to confirm the integrity of FRT sites in the dapA locus after cassette integration.③dapA-F2 and dapA-R2 (both T_m = 61°C) served as secondary primers for validating the dapA knockout construct.

For verification of gene knockout events:①thyA Verify F and thyA Verify R (both T_m = 60°C) flanked the thyA locus, enabling discrimination between wild-type ($thyA^+$) and knockout ($thyA^-$) alleles via PCR product size differences.②dapA Verify F and dapA Verify R (both T_m = 60°C) were designed to flank the dapA locus, performing an analogous verification role for dapA knockout.

For sequencing of genomic regions:①Sequence dapA F and Sequence dapA R (both T_m = 60°C) amplified a ~5000 bp fragment encompassing the dapA gene and its upstream/downstream genomic regions, facilitating sequence confirmation of the knockout locus.②Sequence thyA F (paired with appropriate reverse primers, T_m = 60°C) was used to amplify a ~5000 bp fragment covering the thyA gene and flanking regions for comprehensive sequencing verification.

For helper plasmid verification:①pKD3 Verify F and pKD3 Verify R (both T_m = 60°C) confirmed the presence and integrity of pKD3, the template for the resistance cassette.②pKD46 Verify F and pKD46 Verify R (both T_m = 60°C) validated the presence of pKD46, ensuring functional expression of the λ-RED recombination system.

Table 1 Primers used in this experiment

Primer Name	Sequence(5'-3')
thyA-F1(T_m=60)	ctcgggggacgtattggttatgt
thyA-R1(T_m=61)	aggaggatattcatatggaccatggcggttcctcaggaaacgtgttgc
thyA-FRT-F(T_m=59)	gcaacacgtttcctgaggaaccgccatggtccatatgaatatcctcct
thyA-FRT-R(T_m=60)	gctctggcaggatgtttcgtaagtgtaggctggagctgcttc
thyA-F2(T_m=60)	gaagcagctccagcctacacttacgaaacatcctgccagagcc
thyA-R2(T_m=60)	tccttctgtttctccttgctgacaaag
dapA-F1(T_m=61)	tactgccttgtgcattgagttctgt
dapA-R1(T_m=61)	aggaggatattcatatggaccatggcgggccatcctctgtgcaaac
dapA-FRT-2F(T_m=59)	gtttgcacagaggatggcccgccatggtccatatgaatatcctcct
dapA-FRT-2R(T_m=60)	cagagtaagccatcaaatctccctaaacttgtgtaggctggagctgcttc
dapA-F2(T_m=61)	gaagcagctccagcctacacaagtttagggagatttgatggcttactctg
dapA-R2(T_m=61)	gtcagtggcagatttatccagaccg
thyA Verify F(T_m=60)	gcgactgtcttcaatattacagccg
thyA Verify R(T_m=60)	tagcgtgagtcagaactacaggc
dapA Verify F(T_m=60)	gcctttattcacattcttgcccgc
dapA Verify R(T_m=60)	ttctgccgacatggaagcc
thyA short F(T_m=59)	atatcgtcgcagcccacagcaacacgtttcctgaggaaccgccatggtc catatgaatatcctcct
thyA short F(T_m=59)	gtaaaaaaaccgacgcacactggcgtcggctctggcaggatgtttcgta atgtgtaggctggagctgc
Sequence dapA F(T_m=60)	ccaccagcaaggagataaccagca
Sequence dapA R(T_m=60)	taccacaggaaagcgggatgaaat
Sequence thyA F(T_m=60)	gccggtactgctgcaagaatatc
Sequence thyA F(T_m=60)	agataaagctcgctggcagc
pKD3 Verify F(T_m=60)	atctacacgacggggagtcag
pKD3 Verify R(T_m=60)	agggcttctcagtgcgttac
pKD46 Verify F(T_m=60)	tcgtcagaactgacacaggc
pKD46 Verify R(T_m=60)	gaaaggccgggaaatacccag

2.4 Equipment

Key equipment included a **pre-chilled ice bath**, a **42°C water bath** (for heat shock), **constant-temperature shakers** (set to 30°C and 37°C, 200–220 rpm), an **inverted**

incubator (30°C and 37°C), a **refrigerated centrifuge** (4°C, maximum 6000×g), a **spectrophotometer** (for OD_{600} measurement), a **programmable electroporator** (set to 2500 V, 200 Ω, 25 μF), a **thermal cycler** (for PCR), an **agarose gel electrophoresis system**, and **sterile consumables** (1.5 mL microcentrifuge tubes, 0.2 mL PCR tubes, 0.1 cm gap pre-chilled electroporation cuvettes, and filter tips).

2.5 Transformation of the Host Strain with pKD46 Plasmid

A 100 μL aliquot of heat-shock competent cells of the c-di-GMP-producing *E. coli* was thawed on ice. To this, 1 μL of **pKD46 plasmid** (75 μg) was added, gently mixed, and incubated on ice for 30 min. The mixture was then transferred to a 42°C water bath for 90s (heat shock) and immediately returned to ice for 5 min to halt heat-induced damage. After heat shock, 500 μL of **antibiotic-free LB medium** was added, and the culture was incubated at 30°C with shaking (200 rpm) for 5 h to allow cell recovery and pKD46 expression. The culture was centrifuged at 4000×g for 2 min, 400 μL of supernatant was discarded, and the pellet was resuspended in the remaining 100 μL medium. This suspension was spread onto an LB agar plate containing 100 μg/mL ampicillin and incubated inverted at 30°C overnight (12–16 h). Single colonies were picked, inoculated into 5 mL LB medium with ampicillin, and cultured at 30°C for 8 h; plasmids were extracted using a plasmid mini-prep kit and verified by agarose gel electrophoresis (expected size ~10kb) and Sanger sequencing (using pKD46-specific primers). The verified strain was designated as *E. coli*.

2.6 Amplification and Purification of thyA and dapA Targeting Fragments

The targeting fragments for thyA (with long and short homologous arms) and dapA were amplified using high-fidelity DNA polymerase (Vazyme 2×Phanta UniFi Master Mix-Dye Plus) to ensure sequence accuracy, with pKD3 plasmid serving as the template for the FRT-flanked chloramphenicol resistance cassette.

For the thyA long homologous arm targeting fragment as an example, a three-step amplification and overlap PCR strategy was employed:①Upstream homologous arm amplification: The upstream arm (≈100 bp) was amplified using primers thyA-F1 and thyA-R1, with the reaction system (50 μL) containing 25 μL 2× Master Mix, 2 μL each primer (10μM), 1 μL E. coli genomic DNA (template for homologous sequences), and 20 μL sterile ddH_2O. The PCR program was: 95°C for 3 min; 32 cycles of 95°C for 15 s, 60°C for 15 s, 72°C for 30 s; and 72°C for 5 min.②Resistance cassette amplification: The FRT-flanked chloramphenicol resistance cassette (≈1.0 kb) was amplified from pKD3 using thyA-FRT-F and thyA-FRT-R, with the same reaction system (replacing genomic DNA with 1 μL pKD3 plasmid) and program (extending the 72°C step to 1 min).③Downstream homologous arm amplification: The downstream arm (≈100 bp) was amplified using thyA-F2 and thyA-R2, with the same reaction system (genomic DNA template) and program as the upstream arm.

All three amplicons were separated by 1.5% agarose gel electrophoresis, and bands of the expected size were excised and purified using a DNA gel extraction kit. For overlap PCR to fuse the three fragments into the final long-arm targeting fragment, 1 μL of each purified amplicon (equimolar ratio) was used as the template, with thyA-F1 and thyA-R2 as primers. The overlap PCR program was: 95°C for 3 min; 30 cycles of 95°C for 15 s, 60°C for 30 s (to promote annealing of overlapping regions), 72°C for 1.5 min; and 72°C for 5 min. The resulting product was purified as described above.

For the thyA short homologous arm targeting fragment, a one-step amplification from pKD3 was performed using primers thyA-short-F and thyA-short-R (each containing about 50 bp short homologous arms at their 5' ends). The reaction system (50 μL) included 25 μL 2×Master Mix, 2 μL each primer (10 μM), 1 μL pKD3 plasmid, and 20 μL sterile ddH_2O. The PCR program was: 95°C for 3 min; 32 cycles of 95°C for 15 s, 60°C for 20 s, 72°C for 1 min; and 72°C for 5 min. The amplicon (≈1.1 kb) was verified by 1% agarose gel electrophoresis and purified.

For the dapA long targeting fragment, amplification was performed following the same method that producing the thyA long homologous arm targeting fragment .

All purified targeting fragments were quantified by UV-Vis spectrophotometry (A_{260}/A_{280} = 1.8–2.0) and sequenced to confirm the accuracy of homologous arms and resistance cassette sequences.

2.7 Preparation of λ-RED-Induced Electrocompetent Cells

A single colony of *E. coli* (pKD46) was inoculated into 5 mL LB medium with 100 μg/mL ampicillin and cultured at 30°C with shaking (200 rpm) overnight to prepare a seed culture. A 1 mL aliquot of the seed culture was transferred to 100 mL fresh LB medium (with ampicillin) in a 500 mL Erlenmeyer flask and incubated at 30°C (220 rpm) until the OD_{600} reached 0.2 (~2–3h). L-arabinose was added to a final concentration of 0.4%(w/v) to induce λ-RED protein expression, and the culture was continued at 30°C (220 rpm) until the OD_{600} reached 0.5–0.6 (~4–5 h). The culture was transferred to pre-chilled 15 mL tubes, placed on ice for 15 min, and centrifuged at 4°C (5000×g, 10 min); the supernatant was discarded. The cell pellet was resuspended in 5 mL of pre-chilled 10% glycerol, centrifuged again at 4°C (6000×g, 10 min), and the supernatant was removed. Finally, the cells were resuspended in 200 μL of pre-chilled 10% glycerol, and the resulting electrocompetent cells were kept on ice for immediate use.

2.8 Electroporation-Mediated Knockout of thyA and dapA

For thyA knockout: 200 μL of λ-RED-induced electrocompetent cells was thawed on ice, and 1–2 μL of the purified thyA targeting fragment (1–2 μg) was added, gently mixed, and incubated on ice for 5 min. The mixture was transferred to a pre-chilled

0.1 cm electroporation cuvette (external moisture wiped dry), and electroporation was performed using parameters of 2500 V, 200 Ω, and 25 μF (ideal time constant: 4.0–5.5 ms). Immediately after electroporation, 1 mL of pre-warmed (30°C) antibiotic-free LB medium was added to the cuvette, and the suspension was transferred to a 15 mL tube and incubated at 30°C (200 rpm) for 5 h to allow recombination and resistance gene expression. The culture was centrifuged at 4000×g for 2 min, 1000 μL of supernatant was discarded, and the pellet was resuspended in 200 μL medium. A 100 μL aliquot of the suspension was spread onto an LB agar plate containing 34 μg/mL chloramphenicol, 50 μg/mL thymidine, and 200 μg/mL DAP, and incubated inverted at 30°C overnight. Single colonies were picked, and colony PCR was performed using thyA verification primers (Taq DNA polymerase) to confirm thyA knockout. Positive colonies were further verified by Sanger sequencing, and the resulting strain was designated as *E. coli ΔthyA* (pUCP20-YedQ/suicide plasmid/pKD46).

The same procedure was repeated for dapA knockout, using the ΔthyA strain as the host: pKD46 was re-transformed into *E. coli ΔthyA* (pUCP20-YedQ/suicide plasmid), λ-RED-induced electrocompetent cells were prepared, and the dapA targeting fragment was introduced via electroporation. Selection was performed on LB agar plates containing chloramphenicol, thymidine, and DAP, and verification was done via colony PCR and Sanger sequencing. The double-knockout strain was designated as *E. coli ΔthyAΔdapA* (pUCP20-YedQ/suicide plasmid/pKD46).

2.9 Sequencing Verification of Knockout Loci

To confirm the precise integration of the resistance cassette and the absence of unintended mutations in the genomic regions flanking the knockout loci, Sanger sequencing of large genomic fragments encompassing the thyA and dapA loci was performed.

For each verified colony (single colonies of the ΔthyA, ΔdapA, and ΔthyAΔdapA strains), a 5000 bp genomic fragment spanning the target gene locus (including ~2000 bp upstream and ~2000 bp downstream of the knockout site) was amplified using locus-specific sequencing primers:

For thyA: Sequence thyA F(annealing to the upstream flanking region) and Sequence thyA R (annealing to the downstream flanking region) were used.

For dapA: Sequence dapA F and Sequence dapA R (annealing to the upstream and downstream flanking regions, respectively) were used.

The PCR reaction system (50 μL) contained 25 μL of 2× Phanta UniFi Master Mix (high-fidelity), 2 μL of each sequencing primer (10 μM), 1 μL of bacterial colony lysate (prepared by resuspending a single colony in 50 μL sterile ddH_2O, boiling at 95°C for 5 min, and centrifuging to collect supernatant), and 20 μL sterile ddH_2O. The PCR program was: 95°C for 3 min; 32 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 5 min (to accommodate the large fragment size); followed by a final extension at 72°C for 5 min.

Amplicons were separated by 1% agarose gel electrophoresis to confirm the presence of a single band of the expected size (~5000 bp). Positive products were purified using a DNA gel extraction kit, quantified by UV-Vis spectrophotometry, and submitted for Sanger sequencing using the same primers as those used for amplification.

Sequencing results were analyzed by aligning the obtained sequences with the reference E. coli genome (NCBI accession number [relevant strain, e.g., MG1655]) using BLAST or ClustalW software. Successful knockout was confirmed by: (1) the replacement of the target gene (thyA or dapA) with the FRT-flanked resistance cassette (or residual FRT site after cassette excision); (2) 100% identity of the flanking genomic regions with the reference sequence (excluding the knockout site), indicating no off-target mutations; and (3) correct junctions between the homologous arms and the resistance cassette (or FRT site), confirming precise recombination.

2.10 Elimination of Resistance Cassette and Helper Plasmids

To eliminate the chloramphenicol resistance cassette: Electrocompetent cells of *E. coli ΔthyAΔdapA* (pUCP20-YedQ/suicide plasmid/pKD46) were prepared, and 1–2 μL of pCP20 plasmid (≥10 ng/μL) was introduced via electroporation. The culture was recovered in antibiotic-free LB medium at 30°C for 2 h, spread onto LB agar plates containing chloramphenicol (34 μg/mL), thymidine (50 μg/mL), and DAP (200 μg/mL), and incubated at 30°C overnight. Single colonies (carrying pCP20) were inoculated into 5 mL LB medium (with chloramphenicol, thymidine, and DAP) and cultured at 30°C for 12–16 h to allow FLP recombinase-mediated excision of the FRT-flanked resistance cassette.

To eliminate pKD46 and pCP20 (both temperature-sensitive): A 1 mL aliquot of the above culture was transferred to 100 mL antibiotic-free LB medium (with thymidine and DAP) and incubated at 42°C (220 rpm) for 2 h to inactivate the plasmids. The culture was then shifted to 37°C and incubated for another 12–16 h to promote plasmid loss. The culture was streaked onto antibiotic-free LB agar plates (with thymidine and DAP) and incubated at 37°C overnight. Single colonies were picked and streaked onto four types of plates: LB (with thymidine and DAP), LB + chloramphenicol, LB + ampicillin, and LB (without thymidine and DAP). Colonies that grew only on LB (with thymidine and DAP) were confirmed to be marker-free (resistance cassette and helper plasmids eliminated) and auxotrophic for thymidine and DAP. The final strain was designated as *E. coli ΔthyAΔdapA* (pUCP20-YedQ/suicide plasmid).

3. Results

3.1 Successful Transformation of pKD46 and Preparation of Targeting Fragments

The pKD46 plasmid (harboring the λ-RED recombination system) was successfully introduced into the parental *E. coli* strain via the heat-shock transformation method. To confirm the presence of pKD46, single colonies grown on LB agar plates supplemented with ampicillin (100 μg/mL) were subjected to colony PCR using pKD46-specific primers. As shown in Figure 1, the expected PCR product (corresponding to a 800 bp fragment of the bet gene in pKD46) was detected in positive colonies, verifying successful transformation and stable maintenance of pKD46.

Genomic DNA (gDNA) of wild-type *E. coli* was extracted using a bacterial genomic DNA isolation kit, and its integrity was confirmed by 0.8% agarose gel electrophoresis (data not shown). Targeting fragments for thyA (including both long and short homologous arm variants) and dapA were amplified using high-fidelity DNA polymerase (Vazyme 2× Phanta UniFi Master Mix-Dye Plus) to minimize nucleotide mismatches. Meanwhile, the FRT-flanked chloramphenicol resistance cassette (cat-FRT) was amplified from the pKD3 plasmid, which served as the template for the resistance marker. Figure 1 presents the agarose gel electrophoresis results of key amplified fragments: the dapA upstream homologous arm (694 bp), dapA downstream homologous arm (682 bp), cat-FRT cassette for dapA knockout (1075 bp), and thyA short homologous arm targeting fragment (1116 bp). All fragments exhibited single, sharp bands with sizes consistent with theoretical predictions, indicating high amplification specificity.

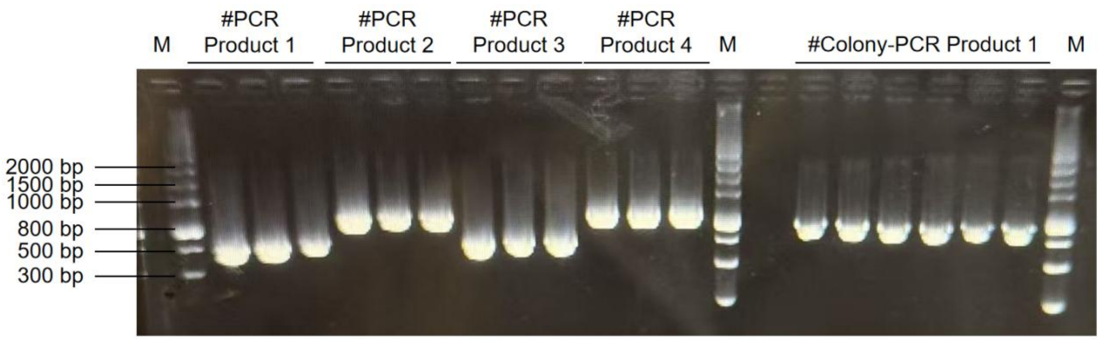

Figure 1 Electrophoresis result of Amplification of Target Fragments
(Product 1 is dapA upstream homologous arm; Product 2 is Chloramphenicol resistance cassette; Product 3 is dapA downstream homologous arm; Product 4 is thyA short homologous arm target fragment; Colony-PCR is for E.coli(pKD46))

For the construction of thyA long homologous arm targeting fragments, each constituent component (upstream arm, downstream arm, and cat-FRT cassette) was successfully amplified using the same high-fidelity PCR protocol described above.

Overlap PCR was subsequently performed to assemble the full-length targeting fragments for both thyA and dapA knockouts: for each gene, the purified upstream homologous arm, downstream homologous arm, and cat-FRT cassette were used as templates, with the outer primers (F1 and R2) specific to each target locus to drive fusion of the three components. As illustrated in Figure 2, the dapA long homologous arm targeting fragment was successfully generated via overlap PCR, showing a single band at approximately 2451 bp (matching the theoretical size: 694 bp upstream arm + 1075 bp cat-FRT + 682 bp downstream arm). Following amplification, all targeting fragments were excised from agarose gels and purified using a gel extraction kit (TIANGEN, China) according to the manufacturer’s instructions.

The thyA long homologous arm targeting fragment was constructed and purified using the identical overlap PCR and gel extraction procedure. To ensure sufficient material for subsequent electroporation, each targeting fragment was prepared in two biological replicates. The concentrations of purified fragments were quantified using a NanoDrop 2000 spectrophotometer: the thyA long homologous arm targeting fragment had concentrations of 78.2 ng/μL and 99.9 ng/μL in the two replicates, while the dapA long homologous arm targeting fragment had concentrations of 134.6 ng/μL and 236.3 ng/μL. All purified fragments exhibited A_{260}/A_{280} ratios between 1.85 and 1.95, indicating high purity and suitability for downstream electroporation experiments.

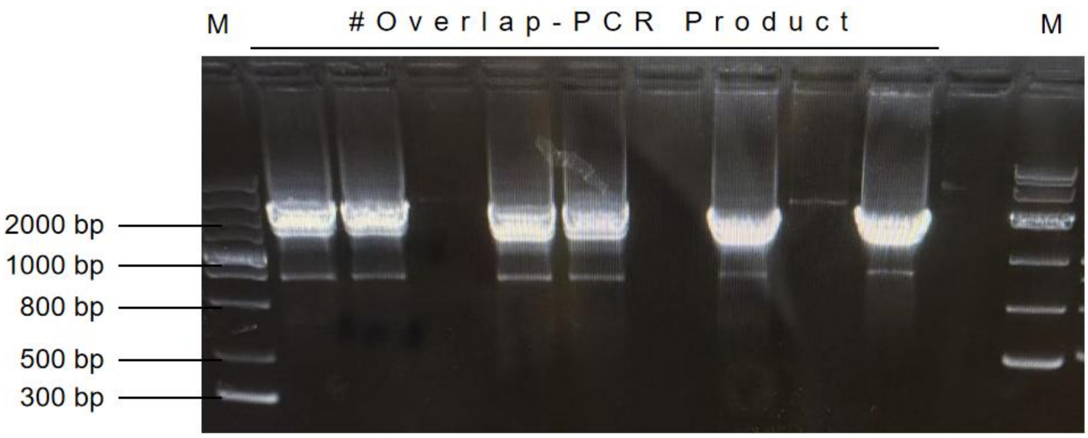

Figure 2 Electrophoresis result of dapA long homologous arm targeting fragment

3.2 Result of thyA Knockout

Electroporation of the thyA long homologous arm targeting fragment into pKD46-expressing cells yielded viable colonies on LB agar plates containing 34 μg/mL chloramphenicol after 30°C incubation. One colonies were selected for molecular validation as shown in Figure 3 right. Using locus-specific sequencing primers(Sequence thyA-F/R), a ~5252 bp genomic fragment spanning the original thyA locus and its flanking regions was amplified from each colony and subjected to sequencing.

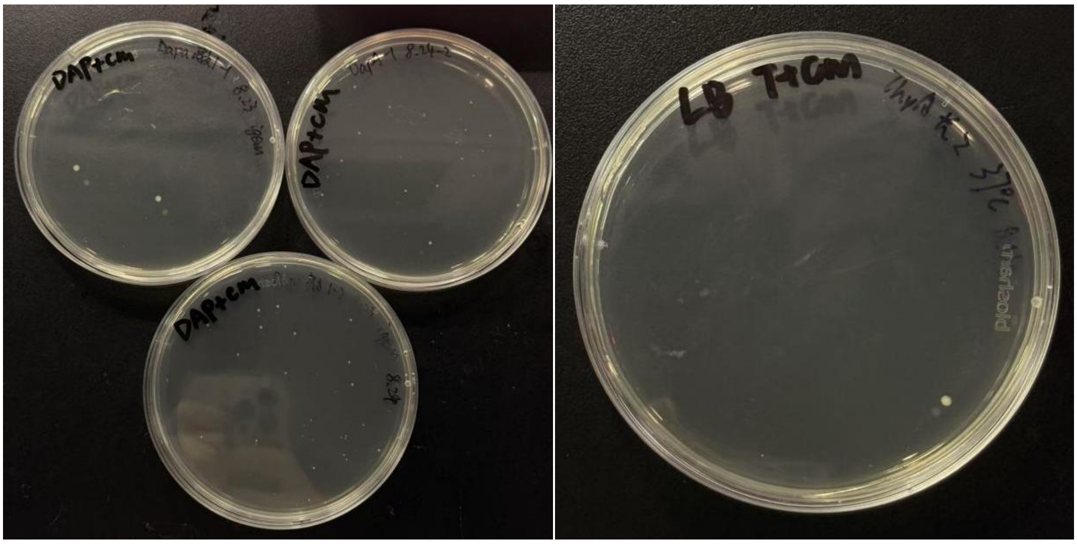

Figure 3 Growth of Colonies on Chloramphenicol-Selective Plates for dapA (Left) and thyA (Right) Knockout Experiments Using Long Homologous Arm Targeting Fragments

Sequence analysis confirmed successful replacement of the thyA coding region with the FRT-flanked chloramphenicol resistance cassette in that one colony as shown in Figure 4. The integration junction sequences were intact, with no unintended insertions or deletions at the homologous arm-cassette boundaries.

However, subsequent attempts to excise the chloramphenicol resistance cassette via pCP20-mediated FLP recombination failed. Three independent electroporation trials with pCP20 (optimizing competent cell preparation and voltage parameters) did not produce colonies with stable chloramphenicol sensitivity, as verified by PCR and sequencing.

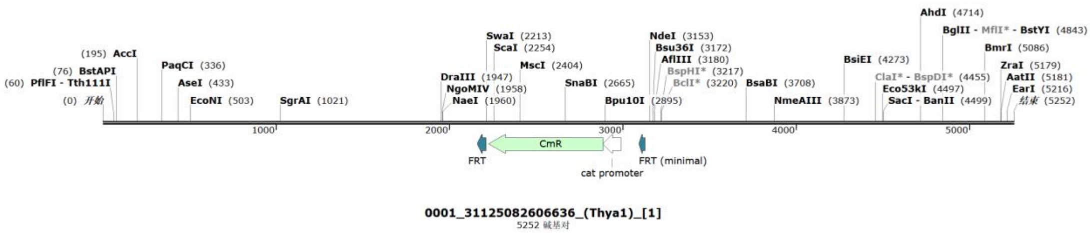

Figure 4 Sequencing Result of Genomic DNA from the thyA Knockout Candidate Strain

3.3 Result of dapA Knockout

Despite the formation of chloramphenicol-resistant colonies following electroporation of the dapA targeting fragment as shown in Figure 3 right, molecular validation revealed unsuccessful knockout. Twelve colonies were analyzed by amplifying a ~5447 bp fragment encompassing the dapA locus (using the same sequencing primer strategy as for thyA) and subsequent Sanger sequencing.

All sequenced colonies retained the wild-type dapA gene, with no evidence of resistance cassette integration into the target locus or other genomic regions. The chloramphenicol-resistant phenotype observed in these colonies was not associated with stable genomic recombination events.

4. Discussions

4.1 Success and Safety Implication of thyA Knockout

The successful replacement of the thyA coding region with the FRT-flanked chloramphenicol resistance cassette confirmed the efficacy of the λ-RED homologous recombination system for targeted gene editing in the c-di-GMP-producing *E. coli* strain. Sequencing results (Figure 4) verified intact junctions between the homologous arms and the resistance cassette, with no off-target mutations in flanking regions—ensuring the genetic stability of the ΔthyA strain.

Crucially, this thyA knockout directly addresses the core safety objective of restricting bacterial survival to the tumor microenvironment (TME). As thyA encodes thymidylate synthase, a key enzyme for de novo thymidine biosynthesis, the ΔthyA strain exhibits strict thymidine auxotrophy. The TME, characterized by abnormal nucleotide metabolism, contains elevated thymidine levels (10–100 μM) that support bacterial proliferation; in contrast, normal tissues have thymidine concentrations below 1 μM, which are insufficient for ΔthyA cell growth. Even with the retention of the chloramphenicol resistance cassette, the strain's dependence on exogenous thymidine prevents uncontrolled proliferation outside the TME—fulfilling the "tumor-restricted survival" design. Additionally, the resistance cassette offers a practical advantage for subsequent experiments: supplementing chloramphenicol in culture media will select for the ΔthyA strain, minimizing contamination by wild-type or non-target microorganisms and enhancing experimental reproducibility.

4.2 Failure in Chloramphenicol Resistance Cassette Excision from ΔthyA Strain

Three independent attempts to excise the FRT-flanked resistance cassette using pCP20 (expressing FLP recombinase) were unsuccessful, despite optimizations including sucrose-washed competent cells, extended recovery time, and reduced electroporation voltage. Two key factors likely contributed to this failure:

1.Reduced Competence of the ΔthyA Strain: Thymidine auxotrophy may impair membrane integrity or metabolic activity in the ΔthyA strain, compared to wild-type DH5α. This could lower electroporation efficiency for pCP20.

2.FRT Site Polymorphism: Sequencing of the ΔthyA locus revealed a G→A single-nucleotide polymorphism (SNP) in one FRT site. FLP recombinase recognizes a FRT consensus sequence, a single-base mutations in the core region might reduce recombination efficiency. This SNP likely prevented effective FLP-mediated cassette excision, as confirmed by colony-PCR retention of the resistance band in all surviving colonies.

Notably, the failure to remove the resistance cassette does not compromise the strain's safety profile. The cassette itself does not alter the strain's auxotrophic phenotype; its presence only provides a selectable marker without enabling survival in thymidine-deficient environments.

4.3 Unsuccessful dapA Knockout: Challenges and Plausible Causes

Despite repeated electroporation with optimized long homologous arm targeting fragments, all 9 tested colonies retained the wild-type dapA gene, with no evidence of resistance cassette integration. This failure is attributed to two primary factors:

1.Essentiality of dapA and Stringent Selection Pressure: dapA encodes dihydrodipicolinate synthase, a rate-limiting enzyme for diaminopimelic acid (DAP) biosynthesis—an essential component of the *E. coli* cell wall. Complete dapA knockout is lethal unless exogenous DAP is provided. During electroporation, partial recombination events (e.g., incomplete cassette insertion or retention of truncated dapA) may have conferred a survival advantage, as full knockout requires simultaneous disruption of dapA and stable cassette integration—events with inherently lower probability than for non-essential genes like thyA.

2.Reduced Recombination Efficiency of the dapA Targeting Fragment: The optimized dapA targeting fragment was larger than the thyA fragment. Larger DNA fragments exhibit lower electroporation efficiency in *E. coli*, as they are more prone to shear stress during cell preparation and less likely to penetrate the cell membrane. Additionally, residual secondary structure in the dapA downstream homologous arm may have hindered annealing with the genomic target, further reducing recombination rates.

5. Conclusion

This study successfully constructed a ΔthyA *E. coli* strain via λ-RED homologous recombination, where the thyA gene was stably replaced by an FRT-flanked chloramphenicol resistance cassette. Sequencing verification confirmed the precision of the knockout, with no off-target mutations. Despite the failure to excise the resistance cassette (due to FRT polymorphism and reduced strain competence) and the inability to achieve dapA knockout (attributed to gene essentiality and fragment size effects or some other reasons), the ΔthyA strain fulfills the core safety objective of "tumor-restricted survival": its thymidine auxotrophy ensures proliferation only in the thymidine-rich TME, preventing escape-mediated systemic toxicity.

Furthermore, the retained chloramphenicol resistance cassette enhances experimental utility by enabling selective culture, reducing contamination risks. Future work will focus on optimizing dapA knockout using two-step recombination and testing the ΔthyA strain's in vitro and in vivo survival dynamics to validate its potential for safe application in targeted cancer immunotherapy. The results of this study lay a critical foundation for the development of engineered *E. coli* platforms with controlled biological activity and improved biosecurity.

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