The experimental objective in May: Construct and validate E. coli cysteine auxotroph biosensor; knockout 8 genes via electroporation to verify plasmid efficacy.

Week1: 5.1-5.7:

(A)Construct pYB1a-AtCOMT、pYB1a-AtCOMT$^{C296F/Q310L/V314T}$(pYB1a-AtCOMT***)

1.PCR + cut gel recovery

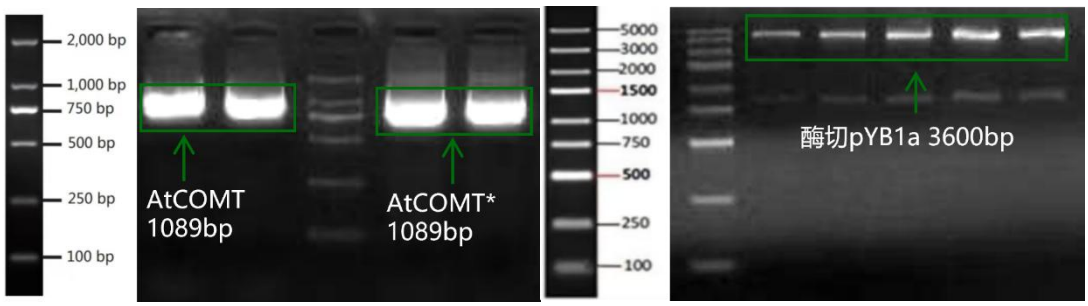
The primer was designed, followed by PCR amplification of the target gene and enzymatic digestion of vector pYB1a

Subsequently, the relevant DNA fragments were assembled using Gibson Assembly methodology, followed by transformation into competent cells. Upon completion of the transformation procedure, polymerase chain reaction (PCR) screening of bacterial colonies was performed.

PCR system (50μL)	
2×Mix	25ul
AtCOMT-F	2ul
AtCOMT-R	2ul
Template	20ng
DDW	20ul

PCR system (50μL)	
2×Mix	25ul
AtCOMT***-F	2ul
AtCOMT***-R	2ul
Template	20ng
DDW	20ul

PCR		
	98℃	5min
	98℃	30s
×30	59℃	30s
	72℃	30s
	72℃	5min
	4℃	∞



2.Connection

Connecting the two fragments using the Gibsion assembly method.

3.Transformation

The ligated system was transferred into DH5α receptor cells, coated with plates, and cultured inverted at 37°C for 12h;

4.Colony PCR

PBF/PBR was chosen as the primer with a theoretical length of 1089bp

5.Plasmid extraction + send for sequencing

The correctly digested protoplasmid was sent for testing, and pYB1a-AtCOMT、pYB1a-AtCOMT***was sequenced correctly.

(B) Concentration of ptarget-ptsG and ptarget-pheA

Concentrated using a vacuum rotary evaporator for 30 minutes, followed by the addition of 10 μL of warm ddH_2O.
Measured concentrations:ptarget-ptsG: 215.135 ng/μL ptarget-pykF: 119.089 ng/μL

(C) Construction of ptarget-pheA Plasmid

Used sgRNA (single guide RNA) to guide Cas9 for site-specific DNA cleavage.
Inserted the pheA gene via PCR.
Inserted pheA into the vector (2118 bp) via PCR.

Purified the PCR products — resulting concentrations: 152.221 ng/μL and 136.968 ng/μL.
Digest the purified products:

System	
纯化产物	1.3μL
DpnI限制酶	0.2μL
rcutsmart 缓冲液	1μL
DDW	7.5μL

PCR system (50μL)	
2×Mix	25μL
pheA--F	2μL
pheA--R	2μL
Template	0.7μL
DDW	20.3μL

Transformed the digested product into DH5α competent cells.Plated the cells and incubated for 12 hours.
Due to no colony growth on the plates, repeated the digestion and transformation steps.
Colonies appeared on the plates. Inoculated colonies and shook for 12 hours.
Preserved cultures and extracted plasmids.
Plasmid concentrations: 54.524 ng/μL and 87.019 ng/μL.

(D)Construction of Donor-pheA U500D500 Plasmid

Used DH5α competent cells as template to amplify U500 and D500 fragments.
Inserted U500 and D500 target fragments into the Donor vector.

1.Performed PCR amplification of Donor, U500, and D500.

PCR system (50μL)	
2×Mix	25μL
U500/D500-F	2μL
U500/D500-R	2μL
Template	1μL
DDW	20μL

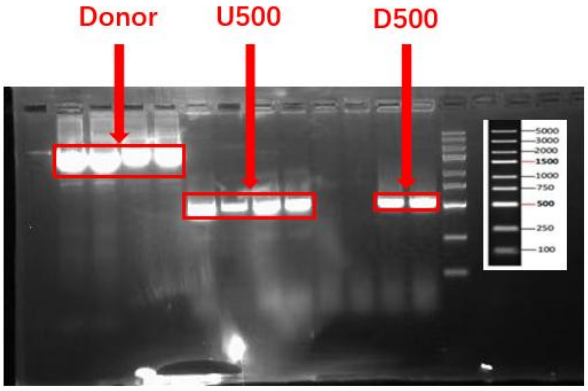
PCR system (50μL)	
2×Mix	25μL
Do-F	2μL
Do-R	2μL
Template	0.12μL
DDW	20.88μL

x 30

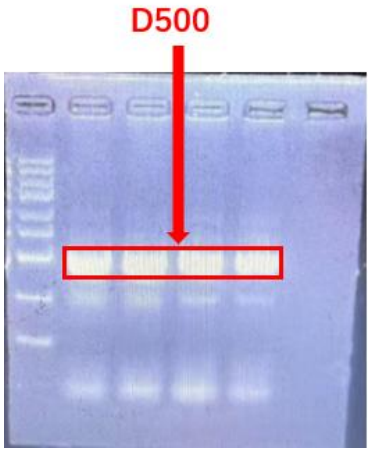
温度（℃）	时间
98	5 min
98	30 s
56	30 s
72	30 s
72	5 min
25	∞

	温度（℃）	时间
	98	5 min
x 30	98	30 s
	55	30 s
	72	67 s
	72	5 min
	25	∞

2.Conducted gel extraction for the three fragments.



Due to low recovery concentration of D500, repeated PCR and gel extraction for D500.



Recovery Concentrations:

Donor: 104.941 ng/μL

U500: 48.267 ng/μL

D500: 40.514 ng/μL 27.812 ng/μL

3.Gibson Assembly of the three fragments:

Gibson system (10μL)	
2×CEMix	5μL
U500	1μL（稀释）
D500	1μL（稀释）
Donor	1μL（稀释）
DDW	2μL

Gibson	
50℃	15min
4℃	∞

4.Transformation and plating

Due to no colony growth on the plates, repeated the Gibson assembly, transformation, and plating steps.

5. Verification of fragment assembly Result Analysis:

PCR system (10μL)	
Green Mix	5μL
Do-JP-F	0.4μL
Do-JP-R	0.4μL
DDW	4.2μL
菌落	μL

PCR		
98℃	5min	
98℃	30s	×30
59℃	30s	
72℃	15s	
72℃	5min	
25℃	∞	

Result Analysis:

Expected band size: 1008 bp

No bands were observed in colony PCR, suggesting possible failure of Gibson assembly.

(E)Preparation of pykA U500D500 and swf U500D500 Targeting Fragments

PCR cloning of ~500 bp upstream and downstream sequences of the target genes.

Example: Donor-pykA U500D500 — 1000 bp

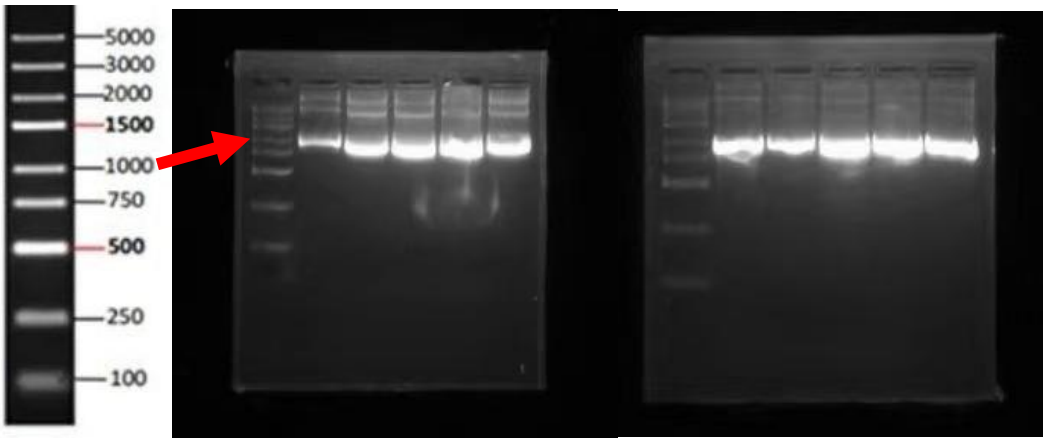
1. PCR ： Donor-pykA U500D500 、Donor-swf U500D500—— 1000 bp

PCR system (50μL)	
2×Mix	25μL
Primer-F	2μL
Primer-R	2μL
Donor Template	1μL（稀释）
DDW	20μL

PCR		
98℃	5min	
98℃	30s	×30
58℃	30s	
72℃	30s	
72℃	5min	
25℃	∞	

2. Gel extraction of pykA and swf U500D500

Result: Bands appeared at the correct positions.



pykA U500D500 **swf U500D500**

pykA U500D500：
269.179 ng/ul

swf U500D500：
181.930 ng/ul

（F） Preparation of Targeting Fragments mtr U500D500 and ppc U500D500

1. PCR

PCR system (50μL)	
2×Mix	25μL
Primer-F	2μL
Primer-R	2μL
Donor Template	1μL（稀释）
DDW	20μL

PCR		
98℃	5min	
98℃	30s	×30
58℃	30s	
62℃	30s	
72℃	5min	
25℃	∞	

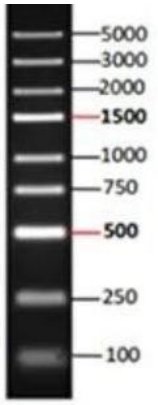
PCR system (50μL)

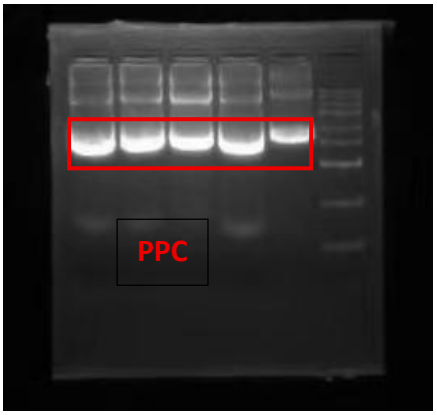
2×Mix	25μL
Primer-F	2μL
Primer-R	2μL
Donor Template	1μL（稀释）
DDW	20μL

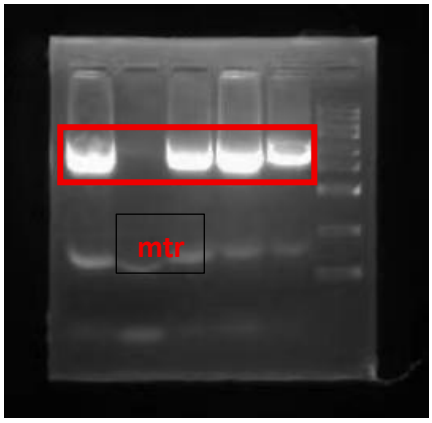
PCR		
98℃	5min	
98℃	30s	×30
58℃	30s	
72℃	36s	
72℃	5min	
25℃	∞	

2. Gel extraction of mtr and ppc U500D500

Result: Bands appeared at the correct positions.







ppc U500D500：
263.966 ng/ul

mtr U500D500：
312.517 ng/ul

（G）Construction of trpE(S40F) Mutant and Fermentation Verification

To systematically enhance tryptophan production in E. coli, this week we initiated two parallel genetic engineering strategies aimed at optimizing the upstream regulation of the tryptophan synthesis pathway (trpEDCBA operon) and the catalytic activity of key enzymes. Specific objectives included:

Construction of promoter YB1a-trpEDCBA: Replacing the original arabinose-inducible promoter PBAD with a medium-strength constitutive promoter P23119 in the plasmid pYB1a-P23119-trpEDCBA.

Construction of a feedback-insensitive mutant plasmid pYB1a-P23119-trpES40FDCBA: Based on the new promoter, introducing a point mutation (S40F) in the key rate-limiting enzyme TrpE to alleviate feedback inhibition by the end product.

1.Fragment Recovery

Amplified pYB1a, trpED, and trpCBA via PCR, followed by product recovery. PCR system:

PCR system (50μL)	
2×Mix	25ul
Primer-F	2ul
Primer-R	2ul
Template	50ng
DDW	20ul

2.Gibson Assembly

Performed Gibson assembly on the recovered fragments. System:

Gibson system (10μL)	
C116 enzyme	25ul
TrpED	0.4ul
TrpCBA	0.5ul
pYB1a	1.5ul
DDW	3ul

3.Chemical Transformation

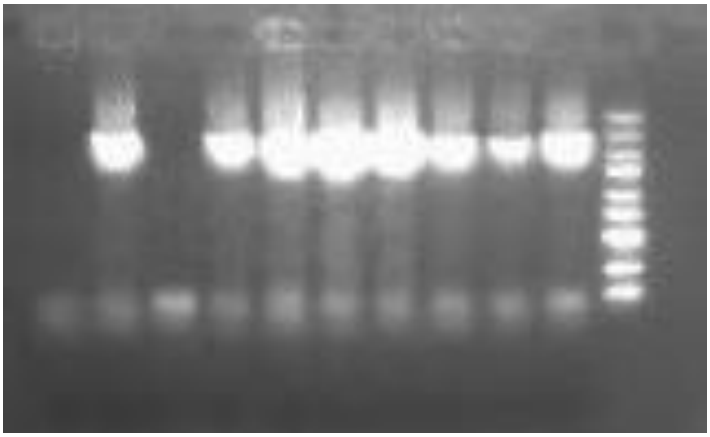
Transformed the digested PCR product into DH5α competent cells. The transformed cells were plated on LB plates containing Amp resistance and incubated overnight at 37°C. Expected resistant single colonies appeared, and individual colonies were selected for plasmid extraction and verification.

4.Colony PCR

Selected 10 colonies for colony PCR. Colony PCR system:

PCR system (10μL)	
2×Hieff	25ul
CE-F	2ul
CE-R	2ul
DDW	4.2ul

The PCR products were analyzed by agarose gel electrophoresis. The positive rate was 80%, and the positive results were confirmed as follows:



5.Sequencing

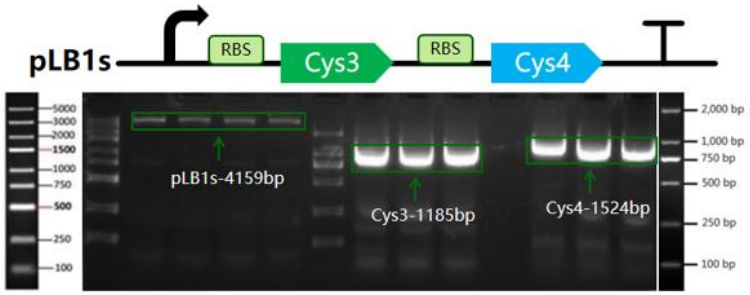
The sequencing results for pYB1a-P23119-trpEDCBA were correct.

Week2: 5.8-5.14:

(A)Construct the pLB1s-Cys3-Cys4 plasmid

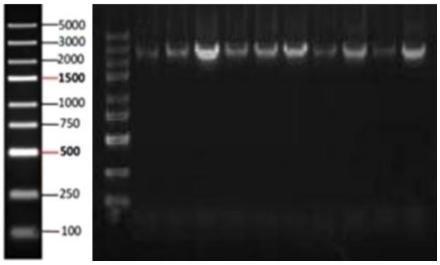
1. Gene amplification and vector processing

Design specific primers, PCR amplified Cys3 and Cys4 genes, and the vector pLB1s was digested to obtain a linearized vector. The obtained PCR bands are as follows：



2.Plasmid ligation and transformation verification

Using the Gibson assembly method, we cloned the amplified Cys3 and Cys4 genes into the linearized pLB1s vector to construct the recombinant plasmid pLB1s-Cys3-Cys4. The plasmid was then transformed into DH5α competent cells and incubated overnight at 37℃°C in slant agar plates. We selected one-third of the 10 appropriately sized single colonies for bacterial plasmid (P) amplification. The confirmed P fragment measured 2730bp. As shown in the results, the plasmid amplification was successful. Subsequently, the plasmid was transformed into an expression plasmid, digested, and sent for sequencing. The construction was successfully completed.

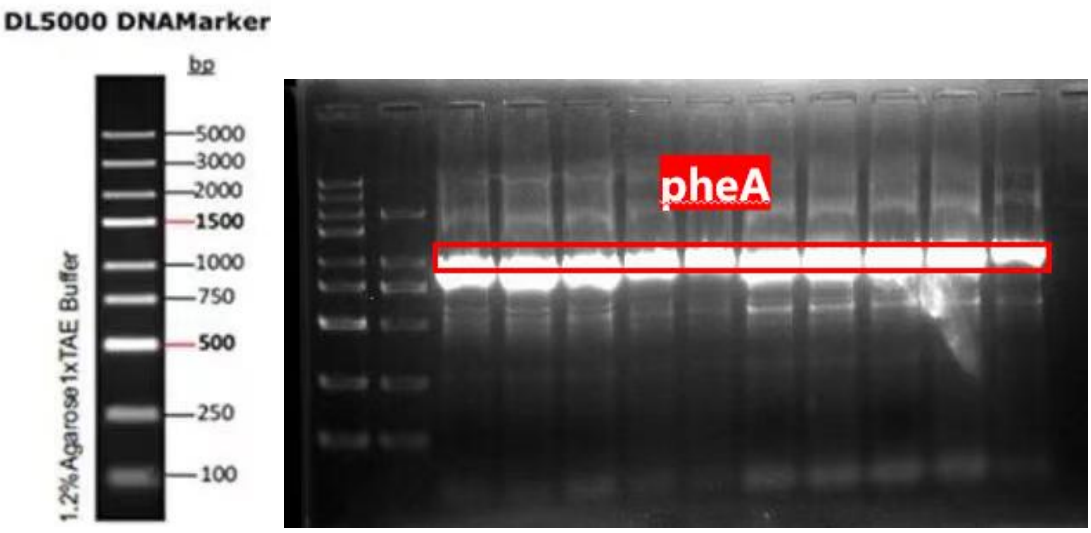


(B)Preparation of pheA, tyrA, ptsG, and pykF Targeting Fragments

1.Assembled U500 and D500 fragments using overlap extension PCR.

2.Gel Extraction

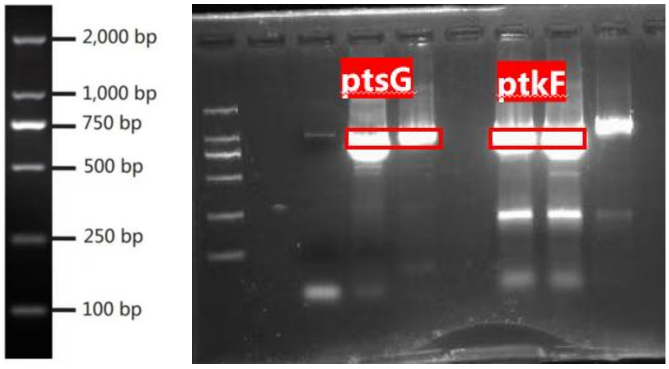
Successfully prepared the pheA targeting fragment with a concentration of 441.904 ng/μL

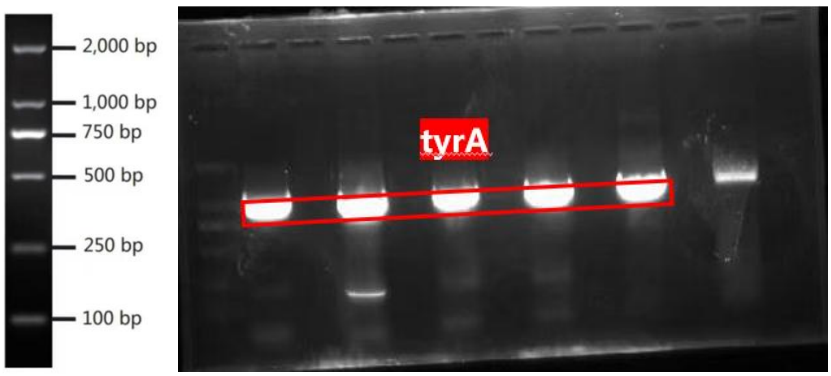


Successfully prepared the tyrA targeting fragment with a concentration of 135.636 ng/μL，

Analysis: Suspecting primer dimer formation from previous attempts, adjusted the PCR program by reducing the annealing temperature by 2°C

Subsequently successfully prepared ptsG and pykF targeting fragments with concentrations of 107.299 ng/μL and 120.650 ng/μL respectively

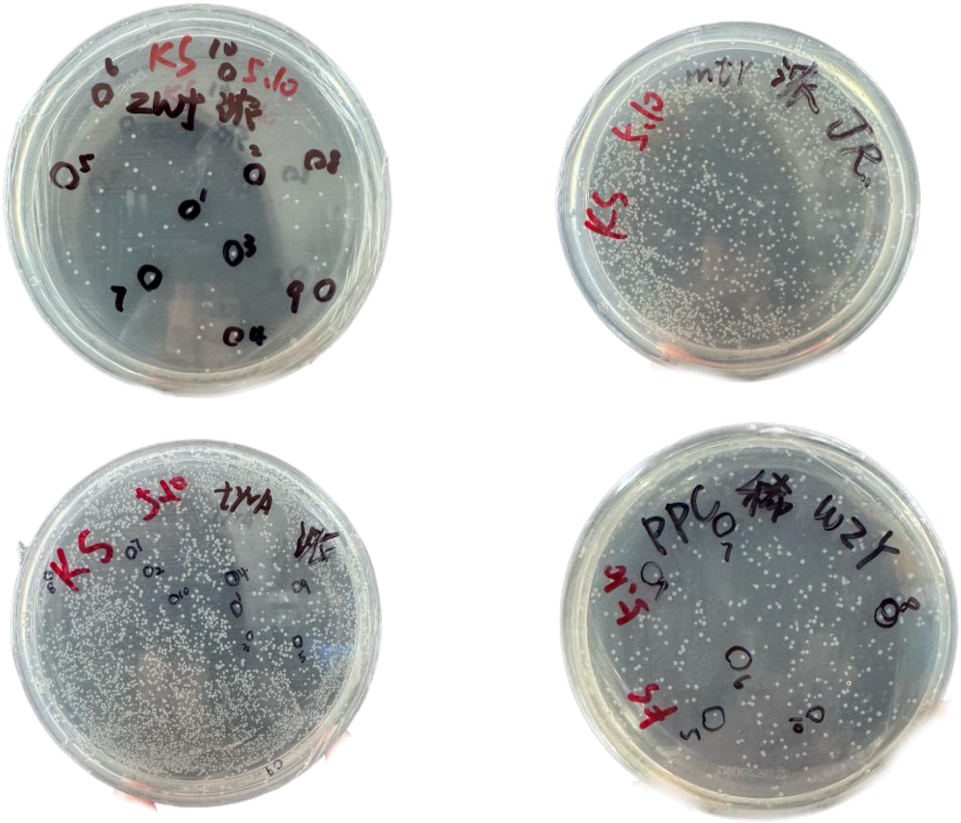




(C)Gene Knockout

1.Performed electroporation for knockout of zwf/mtr/pykA/ppc/pykF/tyrA/ptsG/pheA genes, followed by plating

Results: Colonies grew for tyrA/zwf/mtr/ppc knockouts; No colonies for pykA/pykF/ptsG/pheA knockouts



2.Performed colony PCRtoverifyknockoutfor tyrA/zwf/mtr/ppc

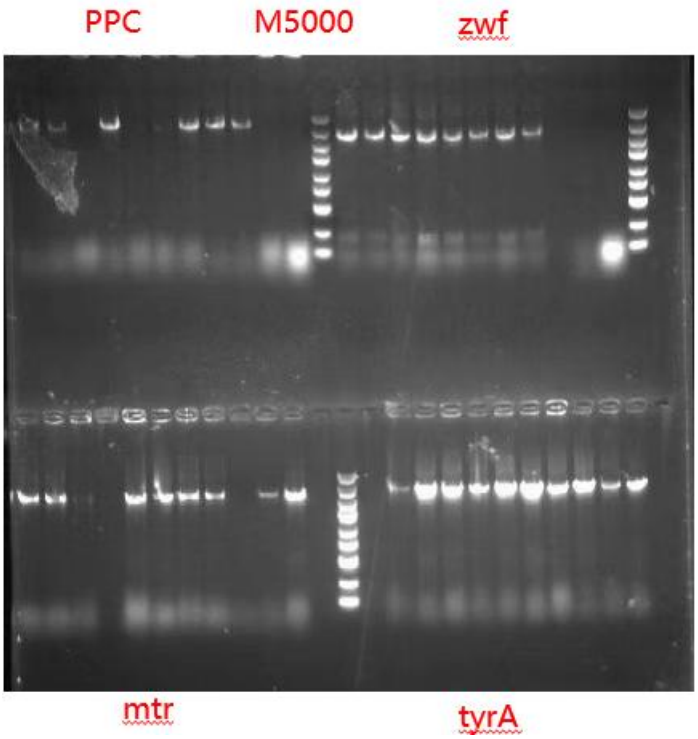
Results: Knockout failed for all verified genes

3.Repeated electroporation and plating for pykA/pykF/ptsG/pheA genes

Results: Still no colony growth

Analysis: Suspected issues with competent cells

4.Repeated electrotransformation for zwf and pykA genes using new batch of competent cells



Week3: 5.15-5.21:

（A） pLB1s-Cys3-Cys4Protein expression validation

1.Transformation

The successfully constructed recombinant plasmid pLB1s-Cys3-Cys4 was respectively transfected into the cysteine nutrition-deficient strain Bw25113ΔCysE and the original strain Bw25113 to obtain two recombinant strains.

（B）Induction and protein detection

1.Sample Preparation

The two recombinant bacterial strains were cultured under induction conditions at 25℃ for 20 hours. The cultures were collected, and the bacterial suspension with a 6 OD unit was measured to obtain the final sample. After centrifugation at 10,000 g for 10 minutes, the supernatant was discarded, and the precipitate was resuspended in 600 μL sterile water. The cells were lysed using an ultrasonic cell disruptor. The lysate was then centrifuged at 10,000 g for 10 minutes to separate the supernatant and sediment. Finally, the precipitate was resuspended in 600 μL sterile water. This procedure completed the sample preparation.

2.point sample

Prepare the protein buffer solution according to the table below. Place the gel in the electrophoresis instrument. After checking for leaks with the buffer solution, mix 16 μL of prepared sample with 4 μL loading buffer. Transfer

the mixed sample into the PCR instrument and heat at 100°C°C for 5 minutes to denature proteins. After cooling, load 16 μL of the mixture into the gel well and add 4 μL of protein marker. The sample loading is now complete.
Concentrating Gel：

12% Lower Gel	2 Gel Units	3 Gel Units	4 Gel Units	5 Gel Units	6 Gel Units	8 Gel Units	10 Gel Units
Solution Components (mL)	10	15	20	25	30	40	50
Water	3.3	4.9	6.6	8.2	9.9	13.2	16.5
30% Acrylamide Solution	4	6	8	10	12	16	20
Tris (pH 8.8)	2.5	3.8	5	6.3	7.5	10	12.5
10% SDS	0.1	0.15	0.2	0.25	0.3	0.4	0.5
10% Ammonium Persulfate	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.004	0.006	0.008	0.01	0.012	0.016	0.02

separation gel：

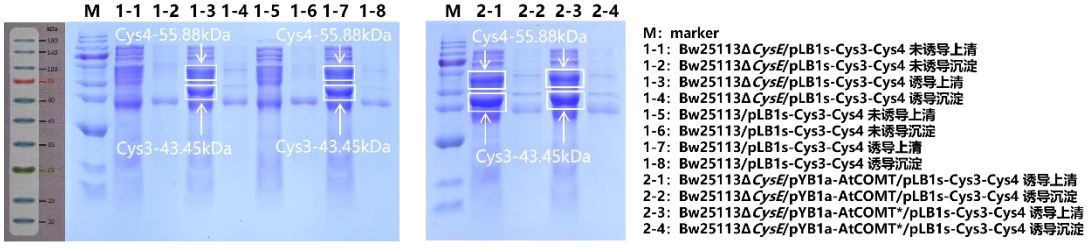
5% Stacking Gel	2 Gel Units	3 Gel Units	4 Gel Units	5 Gel Units	6 Gel Units	8 Gel Units	10 Gel Units	12 Gel Units
Solution Components (mL)	2	3	4	5	6	8	10	12
ddH_2O	1.4	2.1	2.7	3.4	4.1	5.5	6.8	8.1
30% Acrylamide Solution	0.33	0.5	0.67	0.83	1	1.3	1.7	2.01
Tris (pH 8.8)	0.25	0.38	0.5	0.63	0.75	1	1.25	1.5
10% SDS	0.02	0.03	0.04	0.05	0.06	0.08	0.1	0.12
10% Ammonium Persulfate (APS)	0.02	0.03	0.04	0.05	0.06	0.08	0.1	0.12
TEMED	0.002	0.003	0.004	0.005	0.006	0.008	0.01	0.012

3.electrophoresis

Connect the power supply and run the gel at 80 V for 30 minutes, then run it at 120 V for about 1 hour

4.Dyeing and decoloring

Gently remove the gel from the water, then place it in the staining solution. Boil the solution and incubate the gel with heated staining liquid on a small shaker at room temperature for approximately 45 minutes. Rinse the gel thoroughly with distilled water to remove residual staining. Add protein decolorizing solution, heat it to boiling, and decolorize the heated solution on a small shaker at room temperature for 30 minutes. The results are shown in the figure:

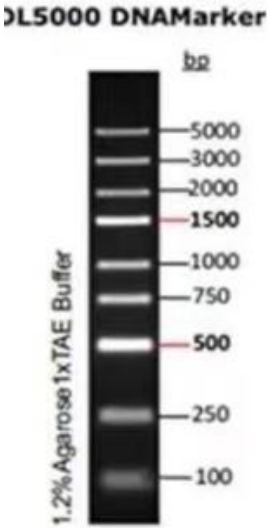


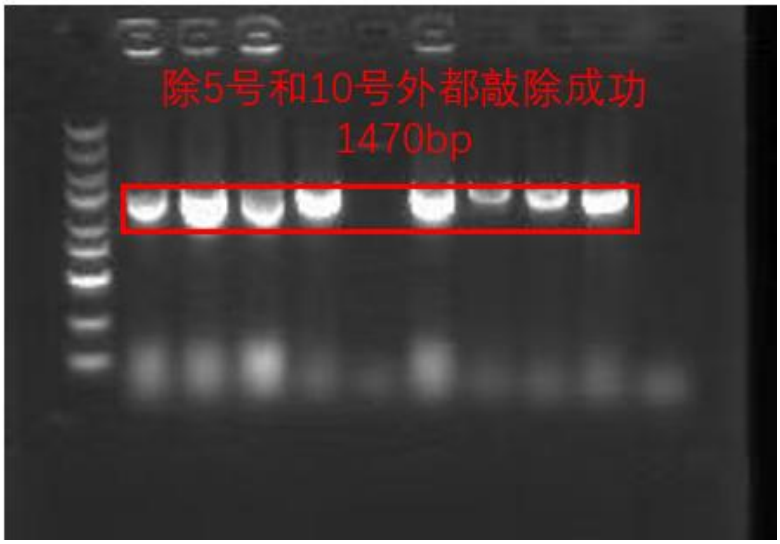
The results showed that the protein was well soluble and could be expressed normally in cells after 25°C induction for 20h.

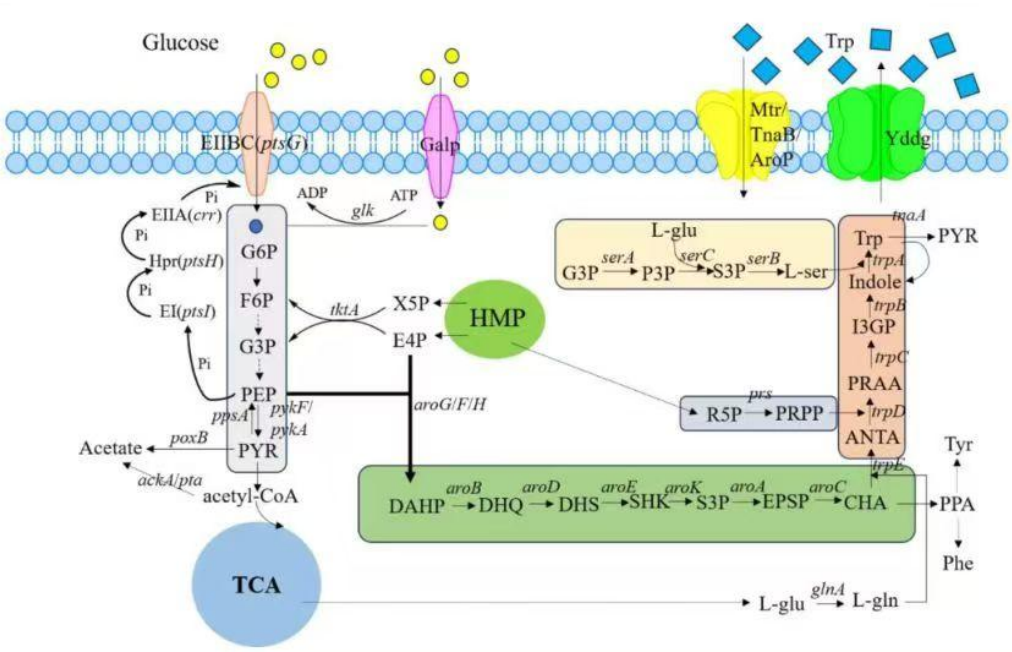
(C) Knockout of the ptsG gene

1.Testing Newly Prepared Competent Cells

Only the ptsG gene was electrotransformed into BW25113ΔtrpR ΔtnaAB to test the efficiency of competent cells.





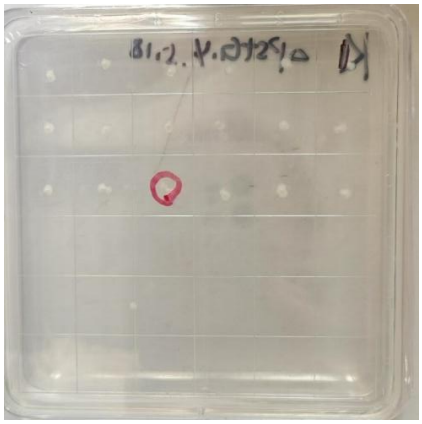


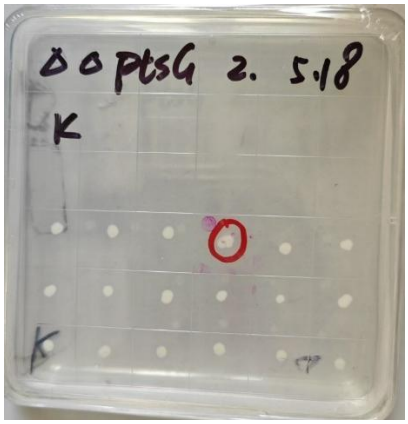
Results: The ptsG gene was successfully knocked out from the genome.

2.Plasmid Curing: Curing of the s-resistant pTarget plasmid

Verification by plating on s-resistant and k-resistant plates.

Results: Colonies grew on both s-resistant and k-resistant plates; the first curing attempt failed.





(D) Knockout of the Remaining 7 Genes

1.Preparation for Electrotransformation: Preparation of insufficient pTarget-mtr, pTarget-tyrA, pTarget-pykA, and pTarget-pykF plasmids

Inoculation and plasmid extraction were performed.

Plasmid concentrations:

pTarget-mtr: 45.137 ng/μL

pTarget-tyrA: 266.054 ng/μL

pTarget-pykA: 72.019 ng/μL

pTarget-pykF: 213.349 ng/μL

2. Electrotransformation of zwf/mtr/pykA/ppc/pykF/tyrA/pheA genes into BW25113ΔtrpR ΔtnaAB.

3.Plating.

(E) Verification of Expression Efficiency After Knocking Out Different Genes

1.Chemical Transformation: Transform trpEDCBA into BW25113ΔtrpR and BW25113.

2.Plating.

3.Inoculation

Original strains:
BW25113
BW25113ΔtrpR
BW25113ΔtrpR Δ tnaAB

Strains with Trp plasmid transformed:
BW25113ΔtrpR Δ tnaAB（no growth）

Strains with pYa-trpEDCBA transformed：
BW25113（no growth）

Strains with pYa-trpEfbrDCBA transformed：
site-directed mutation of trpE)
BW25113ΔtrpR
BW25113ΔtrpR Δ tnaAB

4.Induction: Induction of trpEDCBA and site-directed mutated trpEfbrDCBA expression using arabinose

Induction System Component	Volume
ZY	4.8mL
50x 5052 supplement	100 uL

50x M supplement	100 uL
MgSO4	10 uL
1000x trace element	10 uL
Bacterial suspension	50 uL
arabinose	50 uL
Antibiotic	5 uL

Cultivation in a shaker at 37°C for 2 hours; after adding the inducer, cultivation in a shaker at 25°C for 18 hours.

5.Whole-Cell Catalysis: Using glucose to catalyze the induced enzymes for tryptophan production
Determine the OD_{600} of induced bacterial cells using an ultraviolet spectrophotometer.
Collect 6 OD units of bacterial cells into a centrifuge tube, centrifuge at 4200 rpm for 10 minutes.
Discard the supernatant, resuspend the bacterial cells in M9 conversion broth by gentle pipetting, and transfer to a centrifuge tube for whole-cell catalysis.
Incubate in a shaker at 30°C for 12 hours.

(F) Promoter Replacement and Construction of Site-Directed Mutation S40F

1.PCR

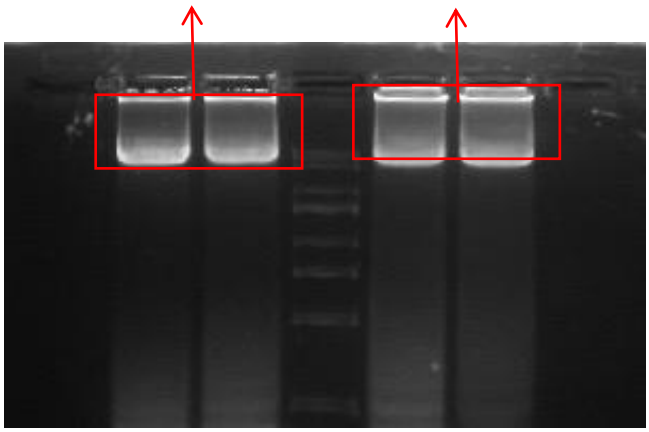
The existing plasmid pYB1a-PBAD-trpEDCBA was used as the template for PCR amplification. Two sets of primers were designed and synthesized: one set for amplifying the trpEDCBA fragment containing the P23119 promoter, and the other set for introducing the S40F mutation codon at the corresponding position of the trpE gene while amplifying P23119-trpEDCBA. The overlap extension PCR strategy was adopted, and the experiment was performed using the following system:

PCR system (50μL)	
2×Mix	25ul
Primer-F	2ul
Primer-R	2ul
Template	50ng
DDW	20ul

2.Agarose Gel Electrophoresis Detection

Agarose gel electrophoresis was performed on the PCR products,
the results showed

pYB1a-P23119-trpE^{S40F}DCBA



the presence of pYB1a-P23119-trpE^{S40F}DCBA and pYB1a-P23119-trpEDCBA.

(3) Digestion and Purification

The PCR products were digested with DpnI to remove the original template plasmid, using the following system:

DpnI system (10μL)	
DNA	200ng
DpnI	1ul
Cutsmart	1ul
DDw	to10ul

(G) Chemical Transformation

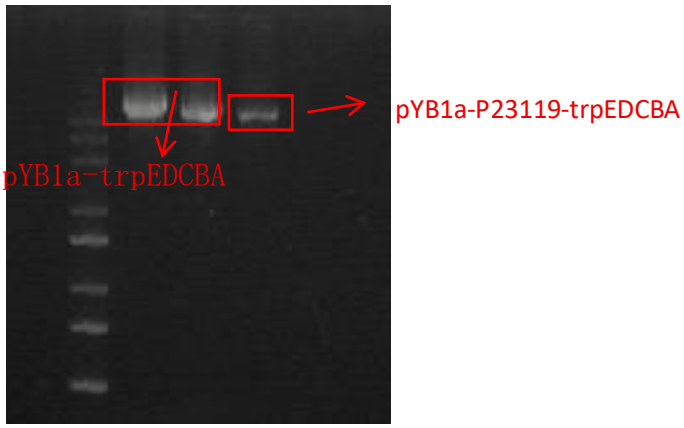
The digested PCR products were transformed into DH5α competent cells. After transformation, the cells were plated on LB agar plates containing Ampicillin (Amp) resistance and incubated overnight at 37°C. Expected resistant single colonies grew on the plates; subsequent steps involved picking single colonies for plasmid extraction and verification.

(H) Colony PCR

Ten colonies were selected from the plates for colony PCR, using the following system:

PCR system (10μL)	
2×Hieff	25ul
CE-F	2ul
CE-R	2ul
DDW	4.2ul

Agarose gel electrophoresis of the PCR products showed that pYB1a-P23119-trpEDCBA had a 1000 bp difference from the original vector, confirming correct construction.



(I) Sequencing

The sequencing result of pYB1a-P23119-trpEDCBA was correct.

(J) Fermentation Test for Tryptophan Synthesis Level

1.Chemical Transformation to Obtain Control Strains:

pYB1a-P23119-trpEDCBA, pYB1a-P23119-tnaES40FDCBA, and PBR322-trpfbrEDCBA-aroGfbr-serAfbr were respectively transformed into BW25113, BW25113ΔtrpR, and BW25113ΔtrpR ΔtnaAB competent cells as control groups.

The transformed cells were plated on LB plates containing Amp or Tetracycline (Tet) resistance and incubated overnight at 37°C, with expected resistant single colonies growing.

2.Chassis Strain

Transformed Plasmid and Corresponding Strain Code

BW25113 **Trp01 000000**	**BWΔtrpR** **Trp02 000000**	**BWΔtrpRΔtnaAB** **Trp03 000000**
pYB1a-P23119-trpEDCBA Trp01 030000	pYB1a-P23119-trpEDCBA Trp02 030000	pYB1a-P23119-trpEDCBA Trp03 030000
pYB1a-P23119-trpE^{S40F}DCBA Trp01 040000	pYB1a-P23119-trpE^{S40F}DCBA Trp02 040000	pYB1a-P23119-trpE^{S40F}DCBA Trp03 040000
PBR322-trp^{fbr}EDCBA-aroG^{fbr}-serA^{fbr} Trp01 050000	PBR322-trp^{fbr}EDCBA-aroG^{fbr}-serA^{fbr} Trp02 050000	PBR322-trp^{fbr}EDCBA-aroG^{fbr}-serA^{fbr} Trp03 050000

3.Streaking: Single colonies were streaked on LB plates containing Amp or Tet resistance to isolate single clones.

Picking Single Clones for Inoculation: Single clones were randomly selected and inoculated into liquid LB medium (with Amp or Tet resistance), followed by cultivation at 37°C for 12 hours.

4.Plating: Bacterial suspension was dipped three times with an inoculating loop from the liquid LB medium and spread on the corresponding resistant LB plates until the plates were uniformly coated, then incubated at 37°C for 12 hours.

5.Inoculation into Seed Medium:

A 250 mL Erlenmeyer flask was used as the container for the seed medium, with the following composition:

Seed culture medium system (15ml)	
Seed medium	13.5ml
Seed glucose	1.5ml
Antibiotic	15ul

Colonies on the plates were scraped off with an inoculating loop and inoculated into the seed medium, followed by cultivation in a shaker at 37°C for 18 hours until the OD value reached 5-6.

6.Inoculation into Fermentation Medium:

6 OD units of bacterial suspension were transferred to a 10 mL EP tube, and the centrifuge was pre-cooled at 4°C, with centrifugation set at 4200 rpm for 10 minutes.

In a clean bench, a 250 mL Erlenmeyer flask was used to prepare the fermentation medium, with the following composition:

Fermentation culture medium system (15ml)	
Fermentation medium	12ml
Seed medium	
Fermentation glucose	1.5ml
Antibiotic	
	1.5ml
	15ul

In the clean bench, the supernatant in the 10 mL EP tube after centrifugation was discarded; 300 μL of the prepared fermentation medium was pipetted into the centrifuged EP tube to resuspend the bacterial cells by pipetting. The liquid in the EP tube was completely poured back into the fermentation medium, and the flask was placed in a shaker at 37°C for cultivation.

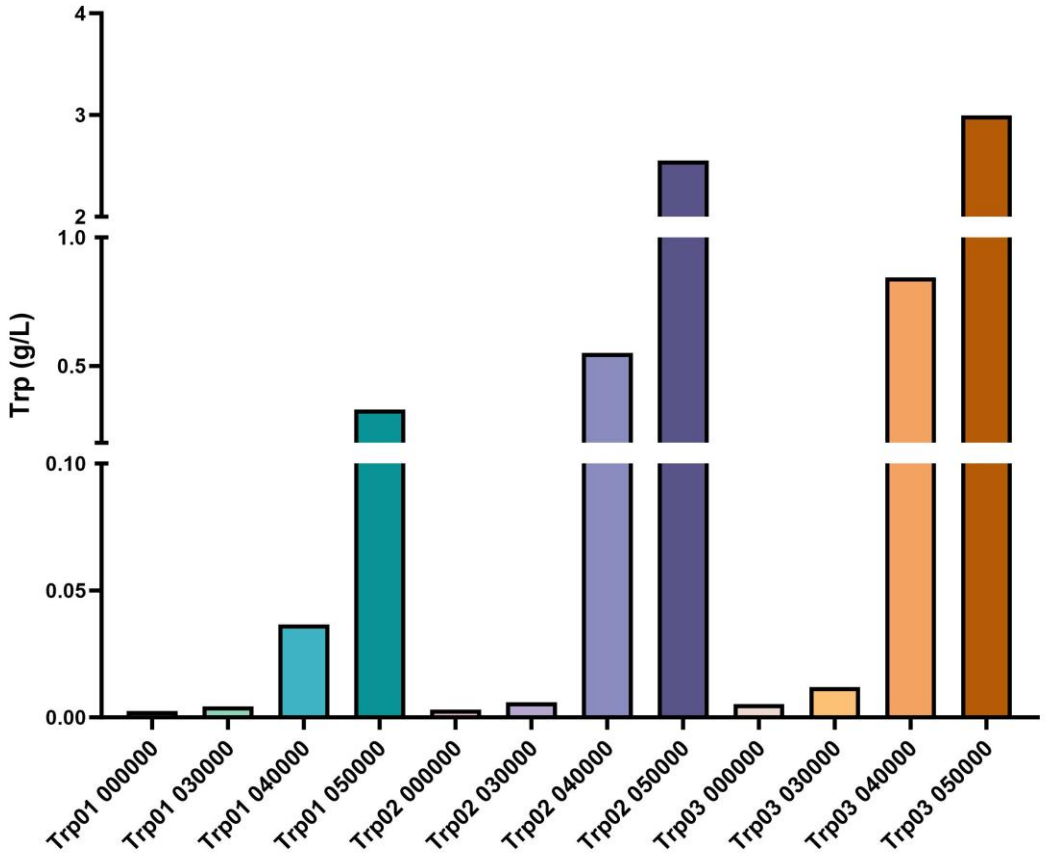
7.OD Value Determination by Spectrophotometer and Sample Preparation for Liquid Chromatography:

At 28 hours after the start of cultivation in the fermentation medium, 500 μL of bacterial suspension was taken from each Erlenmeyer flask containing different strains. Among this, 200 μL was used to determine the OD value with a spectrophotometer. At 28 hours, the OD values indicated that the growth of all strains had basically reached

the stationary phase.

The remaining 300 μL of bacterial suspension was used for sample preparation: centrifugation at 10000×g for 10 minutes, the supernatant was aspirated with a syringe and filtered through a membrane to prepare the sample.

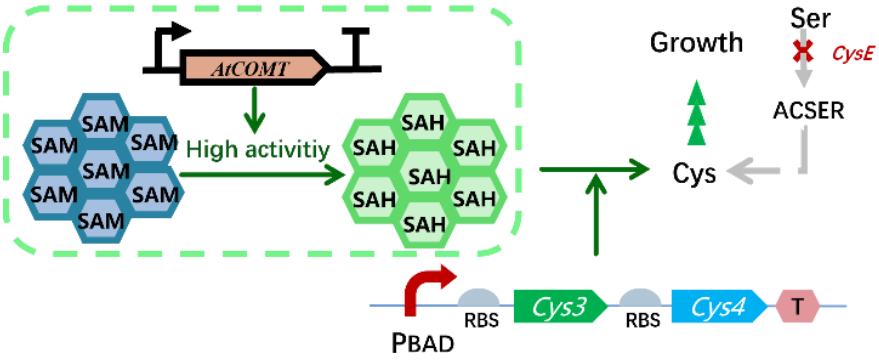
8.High-Performance Liquid Chromatography (HPLC) Detection of Tryptophan Yield.



Week4: 5.22-5.28:

（A）Functional validation of homocysteine nutritional deficiency biosensor

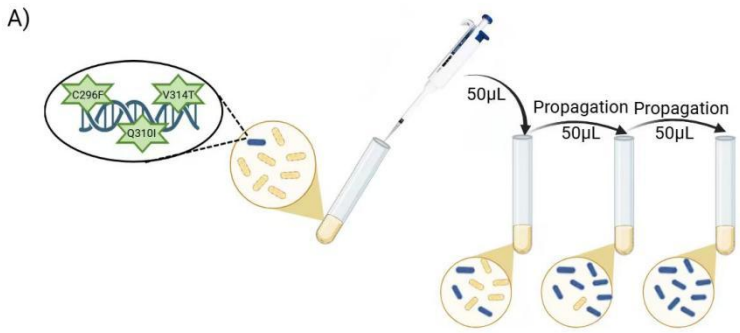
As an essential amino acid for Escherichia coli (E. coli) growth, atecysine (AtCOMT) can only enable SAM methylation through the AtCOMT enzyme in CysE-deficient strains. The synthesis of cysteine proceeds via the Cys3 and Cys4 pathways. Theoretically, higher AtCOMT enzyme activity should improve E. coli's growth performance. Our experimental validation confirmed this hypothesis.



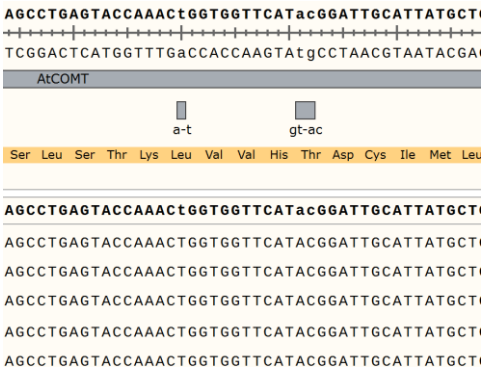
1.Verify the relationship between AtCOMT enzyme activity and E. coli growth

This study utilized wild-type AtCOMT and the enzyme-activated mutant strain AtCOMT***, which exhibits enhanced activity, for comparative analysis of biosensor screening capabilities. When constructed on the pYB1a vector with Bw25113 as the host strain, the results demonstrated that after 50 hours of cultivation, the AtCOMT*** strain exhibited significantly higher growth activity compared to the AtCOMT strain. These findings confirm a positive correlation between AtCOMT enzyme activity and E. coli growth performance.

2.Screening of functional semi-cysteine nutritional deficient biosensors



Stably transform Bw25113ΔCysE/pYB1a-AtCOMT/pLB1s-Cys3-Cys4 into the Bw25113ΔCysE/pYB1a-AtCOMT***/pLB1s-Cys3-Cys4 strain and incubate in 5mL LB liquid medium at 37℃ for 12 hours. Transfer the activated bacterial suspension to M9 medium containing 0.2mM Cys at a ratio of 1:100 (AtCOMT:AtCOMT) and incubate at 30℃ for 16 hours. After overnight cultivation, resuspend the bacterial suspension at 5% concentration and re-inoculate it into fresh M9 medium containing 2mM SAM and 1 mM N-acetyl-5-hydroxytryptamine. Incubate at 30℃ until the culture becomes slightly turbid, then perform subculturing. Conduct streaking on plates after two passages for plasmid DNA sequencing. After sequencing, it was found that all of them were mutants, so the biosensor could be used to screen high enzyme activity mutants.

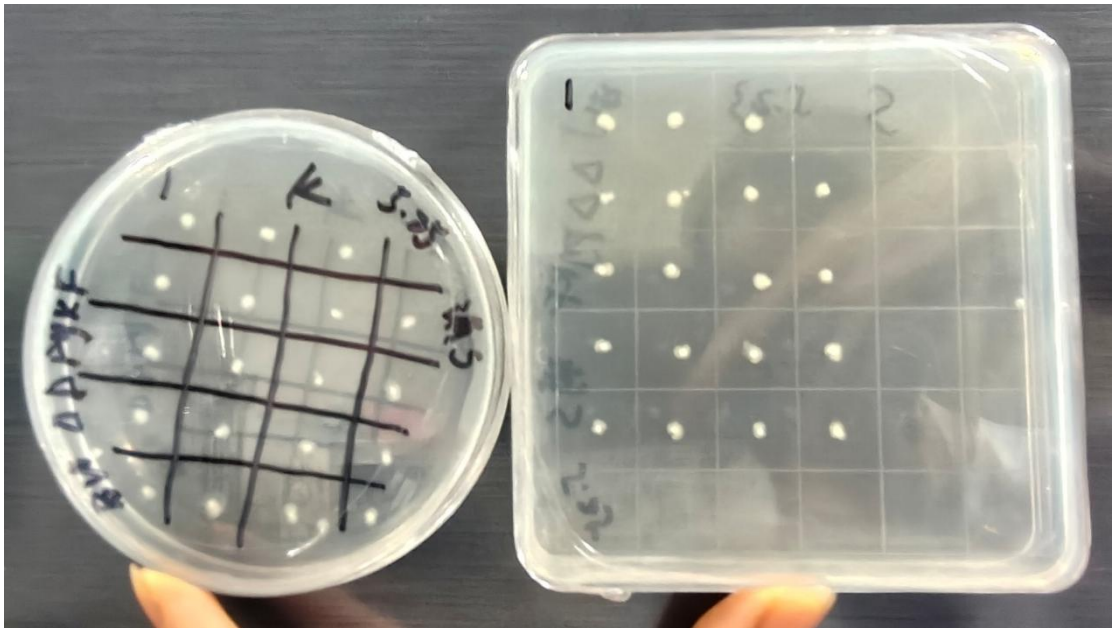


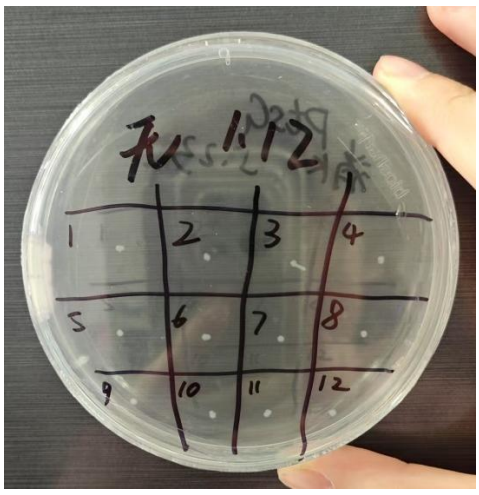
(B) Plasmid Curing

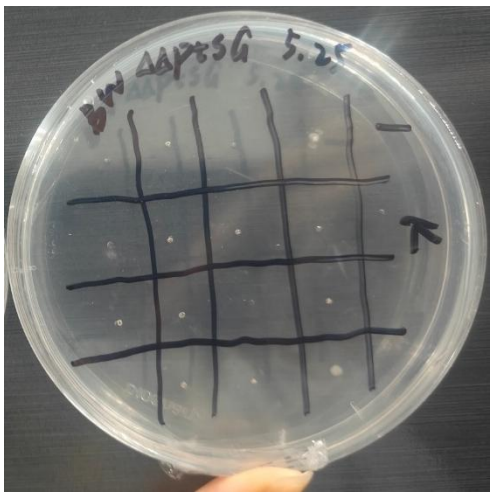
1.Curing of the s-resistant pTarget plasmid from BW25113ΔtrpR ΔtnaAB ΔpykF

Verification by plating on s-resistant and k-resistant plates.

Results: Two curing attempts failed; a third attempt was planned.







2.Curing of the k-resistant Cas9 plasmid from BW25113ΔtrpR ΔtnaAB ΔptsG (from which the s-resistant plasmid had been cured)

Verification by plating on antibiotic-free and k-resistant plates.

Results: The second curing attempt was successful.

(C) Knockout of zwf/pykA Genes

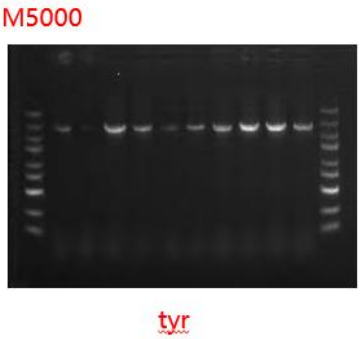
1.Electrotransformation of zwf/pykA genes and plating.

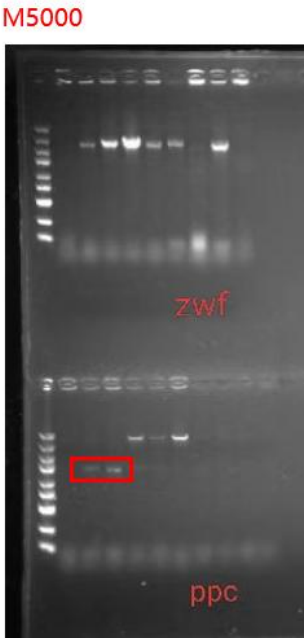
Results: Colonies grew for zwf; contamination occurred in pykA cultures.

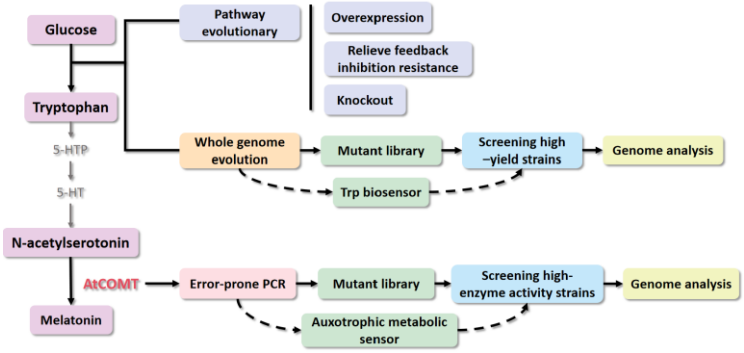
2.Colony PCR Verification for zwf/ppc/tyrA Knockout.

Results: ppc knockout was successful with a positive rate of 20%; zwf/tyrA knockout failed.

Analysis: It was suspected that the 20 bp fragment on the pTarget plasmid had low specificity.






Glucose
Tryptophan
5-HTP
5-HT
N-acetylserotonin
AtCOMT
Melatonin
Pathway evolutionary
Overexpression
Relieve feedback inhibition resistance
Knockout
Whole genome evolution
Mutant library
Screening high –yield strains
Genome analysis
Trp biosensor
Error-prone PCR
Mutant library
Screening high-enzyme activity strains
Genome analysis
Auxotrophic metabolic sensor