The experimental objective in April: Construct and optimize tryptophan biosensors, verify gene knockout effects, and complete CysE knockout and verification to aid strain research.

Week1: 4.1-4.7:

(A) Preparation of electrotransferred sensory states

1. Water-washed organisms

200 μL of bacterial solution was removed from the preserved glycerol tube and added to 5

mL of LB liquid medium. After incubation at 30°C and 200 rpm for about 12 h, the culture was transferred to a new LB medium at 2% inoculum.

2. Induction

After 0.5 h of incubation, add arabinose at a final concentration of 0.2%; incubate at 30°C for about 2 to 2.5 h until the OD600 reaches 0.55-0.6 (either too high or too low will severely affect the efficiency of the electrotransferred sensory state). Remove the culture solution and let it stand on ice for 30 minutes. Pre-cool the centrifuge rotor to -80°C and let stand for 10 minutes.

3. Centrifugation

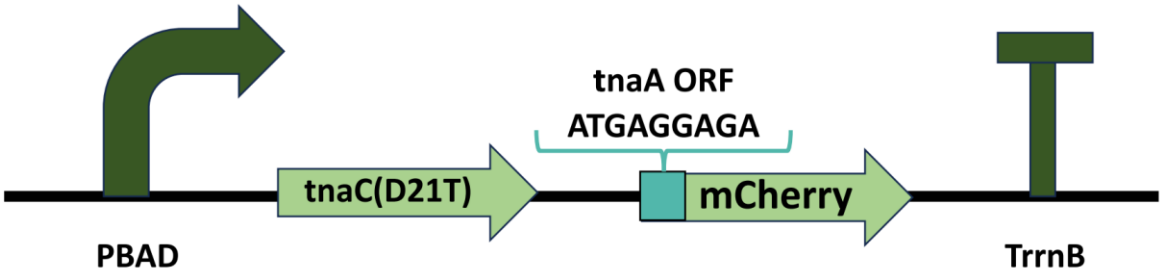
Collect the organisms in pre-cooled centrifuge tubes, kept on ice at all times, and centrifuge at 4000 rpm for 10 minutes; dispense 50 mL per tube. gently aspirate and beat with 18 mL/tube of ice-pre-cooled, sterilised 10% glycerol, kept on ice at all times, and dispense into two 10 mL centrifuge tubes. Centrifuge at 4200 rpm for 10 minutes using a cryogenic centrifuge; immediately after completion of centrifugation, pour off the supernatant and gently lance it, keeping it on ice at all times. Repeat the above steps three times, using 5 mL of ice pre-cooled sterilised 10% glycerol for the first two washes. The last time the organisms were resuspended using 0.25 mL of 10% glycerol and the two tubes were combined into one.

4. Dispensing

Dispense 100 μL into pre-cooled centrifuge tubes, which can be electrotransformed or immediately stored in a -80°C refrigerator, and this sensory state can be stored at -80°C for half a year.

(B)Construct pLB1s-PBAD-tnaC(D21T)-mCherry sensor

1. Plasmid Map



2. Site-directed mutagenesis of tnaC gene by PCR

Using the previously constructed pLB1s-PBAD-tnaC-mCherry plasmid as a template, site-directed mutagenesis was performed by PCR to obtain the pLB1s-PBAD-tnaC(D21T)-mCherry plasmid

PCR system:

pLB1s-PBAD-tnaC(D21T)-mCherry PCR system（50μL）	
pLB1s-PBAD-tnaC(D21T)-mcherry	1μL
D21T -F	2μL
D21T -R	2μL
2×HF Mix	25μL
Ddw	20μL

PCR program:

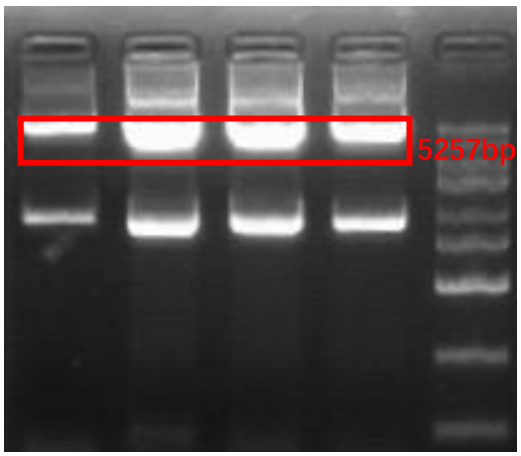
pLB1s-PBAD-tnaC(D21T)-mCherry PCR program		
98℃	5min	× 30
98℃	30s	
55℃	30s	
72℃	150s	
72℃	5min	
25℃	∞	

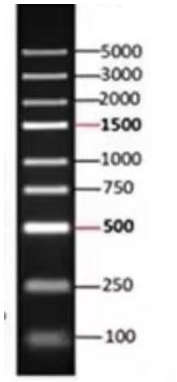
3. Gel extraction of gene fragments

The PCR products were subjected to agarose gel electrophoresis, followed by gel cutting and recovery.

The results are as follows:

pLB1s-PBAD-tnaC(D21T)-mcherry





4. DpnI digestion of pLB1s-PBAD-tnaC(D21T)-mCherry fragment

DpnI digestion reaction system:

DpnIdigestion reaction system（50μL）	
pLB1s-PBAD-tnaC(D21T)-mcherry	5μL
DpnI	1μL
rcutsmart	5μL
Ddw	39μL

DpnIDigestion program：

DpnIDigestion program	
37℃	4h
25℃	∞

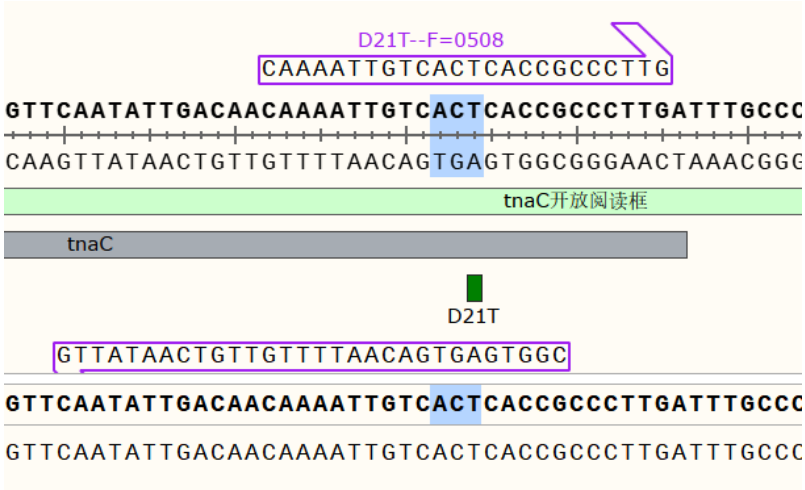
5. Transformation of digested products into DH5α

Using the chemical transformation method, the ligation products were transferred into DH5α competent cells, which were then spread on LB plates containing Str resistance and incubated overnight at 37°C. The expected resistant single colonies grew on the plates, and subsequent

single colonies were selected for colony PCR.

6. Plasmid extraction and sequencing

The sequencing results showed that the D21T site mutation of the tnaC gene was successful. Therefore, the biosensor pLB1s-PBAD-tnaC(D21T)-mCherry was successfully constructed and can be transformed into BW25113 for induced expression and effect testing.。

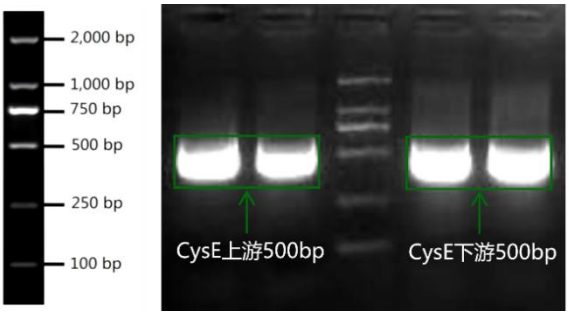


Week2: 4.8-4.14:

(A)Target fragment preparation

1.PCR to obtain target fragments

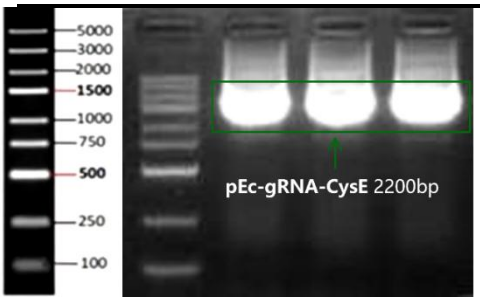
The upstream 500bp and downstream 500bp fragments of CysE gene were amplified by designed primers, and the recovery of the two fragments is shown in the figure：



2. Connections

A 1000bp CysE targeting fragment was constructed by Overlap connection system. The connection system is as follows:

Overlap(50μL)	
CysE-up500bp	50-100ng(0.5μL)
CysE-down500bp	50-100ng(0.5μL)
Top strand primer for the 500bp upstream	2ul
Bottom chain primer for the next 500bp	2ul
PCR 2×Mix	25ul
DDW	20ul



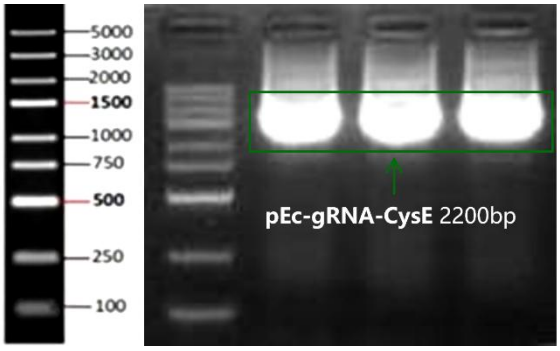
3.Extract plasmid, send for sequencing

Results were verified by sequencing

（B）Construct pEc-gRNA-CysE plasmid

1.PCR to obtain target fragments

The recovery gel is shown in figure.:



2.Digestion

Digestion plasmids, the digestion system is shown in the figure, and the purified plasmids were digested and transferred into DH-5α receptor. After 12h of culture, bacteria were picked to improve the plasmids for sequencing. The sequencing results were correct:

Digestion system(10μL)

DpnI	0.2μl
rcutsmart	1μl
Template DNA	200ng-400ng
DDW	to 10μl

(C) Detecting the impact of trpR and tnaAB gene knockouts on tryptophan production

1. Preparation work

1. 1 BW-ΔtrpR and BW-ΔtrpRΔtnaAB strains have been obtained using the CRISPR-Cas9 method.
2. Preparation of competent cells

① Day 1: Streak the original competent bacteria, pick a single colony and transfer to a test tube containing LB medium, and sterilize the preparation supplies.

② Day 2: Transfer 1ml of bacterial culture from the test tube to 100ml of LB medium, place in a shaker at 30°C. Measure the OD600 after approximately 1-1.5 hours, then measure again according to the turbidity. When the OD600 reaches around 0.2, transfer to a pre-cooled shaker at 16°C and shake until the OD600 is slightly greater than 0.4. Immediately place on ice and let stand for 30 minutes.

③ Pre-cool yellow and blue pipette tips, centrifuge tubes, EP tubes, EP tube racks (place in -20°C freezer), 5 pieces of parafilm, and pre-cool the high-speed centrifuge (4200rpm, 10min, 4°C). Remove the alcohol lamp from the ultra-clean bench and do not use it afterward.

④ After standing, aliquot the LB culture from the Erlenmeyer flask into 4×50ml centrifuge tubes,

approximately 24-25ml per tube. Seal with parafilm, take out and place on ice, centrifuge for 10 minutes. Prepare two ice boxes, place two solutions in them, put into the ultra-clean bench, and sterilize the ultra-clean bench at this time.

⑤ Take pre-cooled blue pipette tips, and pour off the LB medium from the centrifuge tubes in the ultra-clean bench. Add 1.6ml of Activation Solution 1 (80mM $MgCl_2$, 20mM $CaCl_2$) to each tube, disperse the bacterial pellets, then combine into one tube, and let stand on ice for 30 minutes.

⑥ Centrifuge for 10 minutes, sterilize the ultra-clean bench at this time, and return the covered blue pipette tips to the freezer for pre-cooling.

⑦ Take out blue pipette tips, yellow pipette tips, EP tubes and tube racks. Discard the supernatant in the ultra-clean bench, add 3ml of Activation Solution 2 (15% glycerol, 100mM $CaCl_2$), disperse the bacterial pellets, then aliquot 100μl each into EP tubes using cut yellow pipette tips. After aliquoting, place in ice.

⑧ Pre-cool sequencing bags, put the aliquoted competent cells into them, label properly, and store in -80°C.

2. Transformation with overexpression plasmid pYB1a-trpEDCBA

Using the chemical transformation method, the ligation products were transferred into DH5α competent cells, which were then spread on LB plates containing Amp resistance and incubated overnight at 37°C. The expected resistant single colonies grew on the plates, and subsequent single colonies were selected for colony PCR.

3.Induction and whole-cell catalysis

1. 1 Inoculate into ZY5052 auto-induction medium and induce at 25°C for 20 hours. (Arac does not need to be added to the uninduced control group)

ZY5052 Reaction system （5mL）	
ZY	4.8mL
50×M	100μL
50×5052	100μL
1000×elements	10μL
1M $MgSO_4$	10μL
Bacterial	50μL
Antibiotic	5μL
arac	50μL

2. After 20 hours, take it out and place on ice, then perform ultraviolet spectrophotometric measurement.

3. Take the bacterial solution corresponding to 6 OD of bacterial quantity into an EP tube, centrifuge at 4000 rpm for 10 minutes to enrich the bacterial cells, and discard the supernatant.

4. Add 200 μL of M9 to the enriched bacterial cells for resuspension, and incubate at 30°C for 12 hours.

M9 Reaction system（10mL）	
20% Glucose	1mL
1M $CaCl_2$	1μL
1M $MgSO_4$	20μL
Antibiotic	10μL
ddw	Up to 10mL

5. After centrifugation at 10,000g for 10 minutes, take 100 μL of the supernatant and add 900 μL of distilled water, vortex, then filter through a filter membrane and inject into a brown vial.

4.HPLC Detection

1. Detection method:

   Stationary phase: Agilent C18 column (250mm×4.6mm, 5μm, Agilent)

   Mobile phase: 0.3g/L $KHPO_4$ (aqueous solution) mixed with methanol at a volume ratio of 9:1

   UV detection wavelength: 278nm

   Injection volume: 10μL

   Flow rate: 1.0mL/min

Column temperature: 39℃

Product peak time: approximately 25 minutes

2. Detection process

① Flush out bubbles in the pipeline at a flow rate of 5.0mL/min.

② Turn on the infusion pump, pass the mobile phase through the column at a flow rate of 0.7mL/min until the baseline is stable.

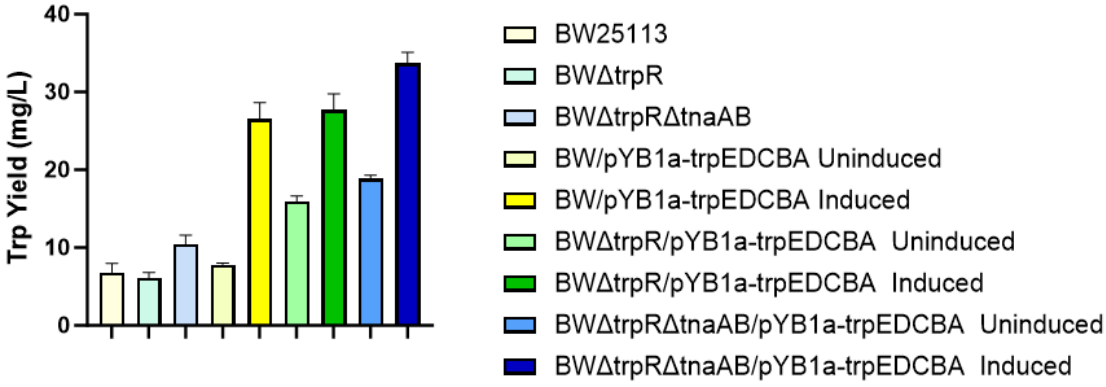
③ Inject different samples into the HPLC system in sequence, with a flow rate of 1.0mL/min and an injection volume of 10μL.

④ Obtain peak areas for data analysis.

3.Data analysis

A total of three replicates were performed. The data (with error values removed) were averaged, and the bar chart is as follows:

Bacterial	Trp Yield (1 组)	Trp Yield (2 组)	Trp Yield (3 组)
BW25113	5.32256257	7.462952501	7.478737087
BWΔtrpR	6.607427912	6.459052799	5.32256257
BWΔtrpRΔtnaAB	11.45013905	9.091921828	10.70194965
BWΔtrpR/pYB1a-trpEDCBA Induced	30.0696373	26.88430774	26.20872744
BWΔtrpRΔtnaAB/pYB1a-trpEDCBA Uninduced	18.54633214	19.17906857	
BWΔtrpRΔtnaAB/pYB1a-trpEDCBA Induced	35.30441669	32.682053	33.06870906

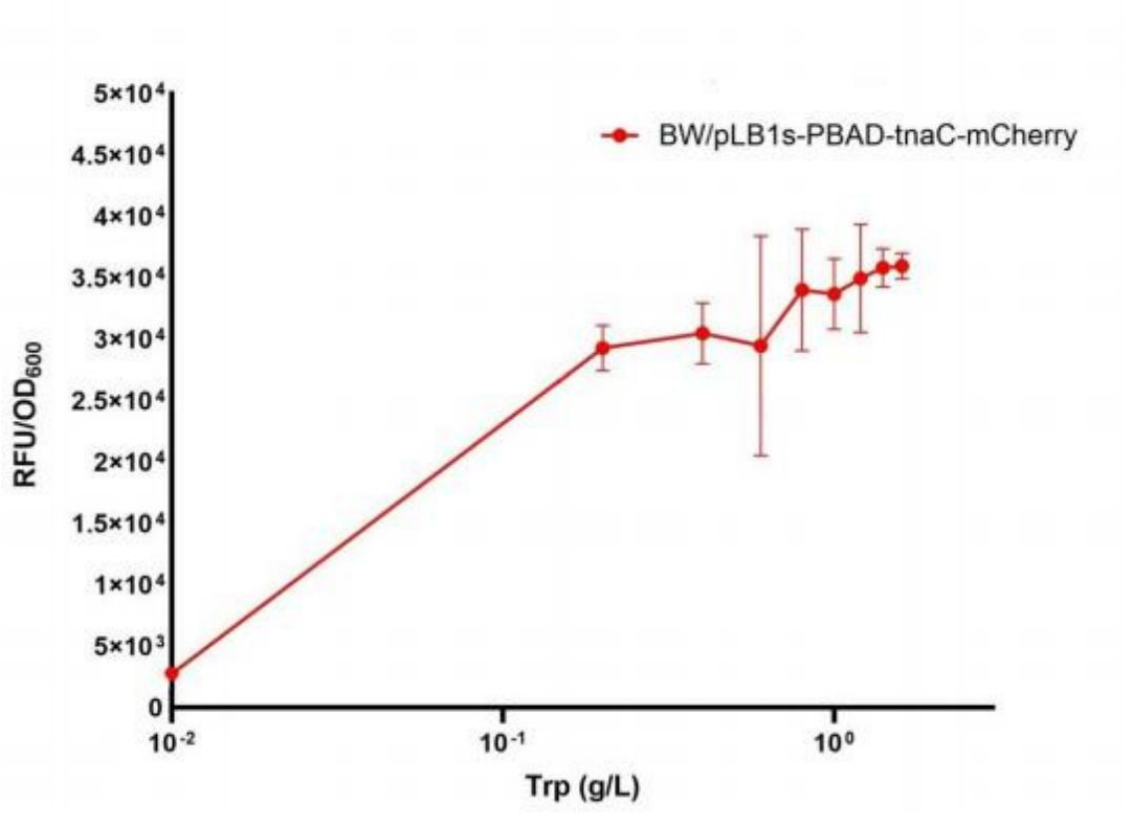


5. Analysis and Discussion

As shown in the figure, without overexpression of trpEDCBA, the tryptophan expression levels of BW25113, BW-ΔtrpR, and BW-ΔtrpRΔtnaAB increased sequentially. After overexpressing trpEDCBA, gene knockout more significantly improved tryptophan production, and BW-ΔtrpRΔtnaAB showed higher yields than BW-Δtrp

6. Data Analysis

Tryptophan(g/L)	RFU/OD600 1 组	RFU/OD600 2 组
0.01	2387.968079	3089.947090
0.2	27986.20690	30596.39390
0.4	28732.52008	32236.92346
0.6	23143.28457	35788.53423
0.8	30511.49931	37507.53873
1.0	31644.16586	35716.68312
1.2	31837.20395	38056.08185
1.4	34734.77157	36920.05114
1.6	36703.35499	35235.37061

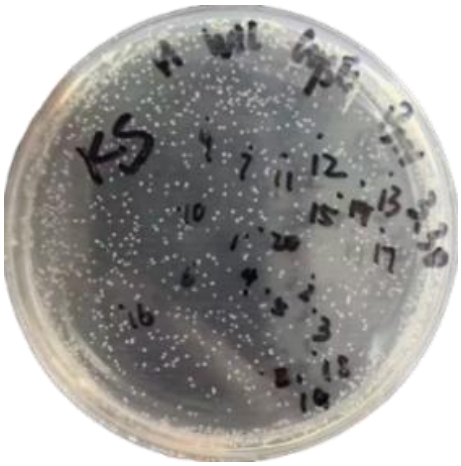


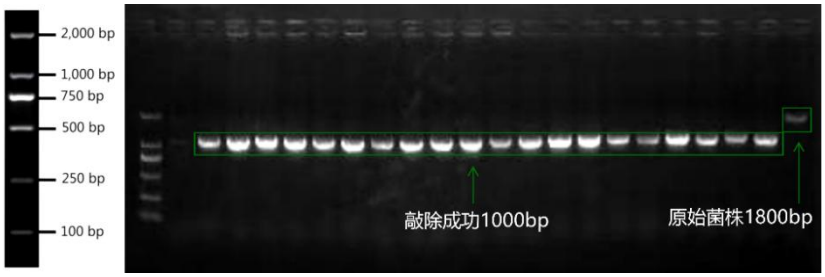
According to the image analysis, the tryptophan response concentration of the pLB1s-PBAD-tnaC-mCherry biosensor is concentrated in the range of 0.01-0.2 g/L. Compared with the pSB1c-BAD-tnaC-mCherry sensor in the previous test, the response effect is slightly better. Therefore, it is planned to further combine the chloramphenicol resistance gene based on pLB1s-PBAD-tnaC-mCherry to construct a growth-coupled sensor.

Week3: 4.15-4.21:

(A) knock-out

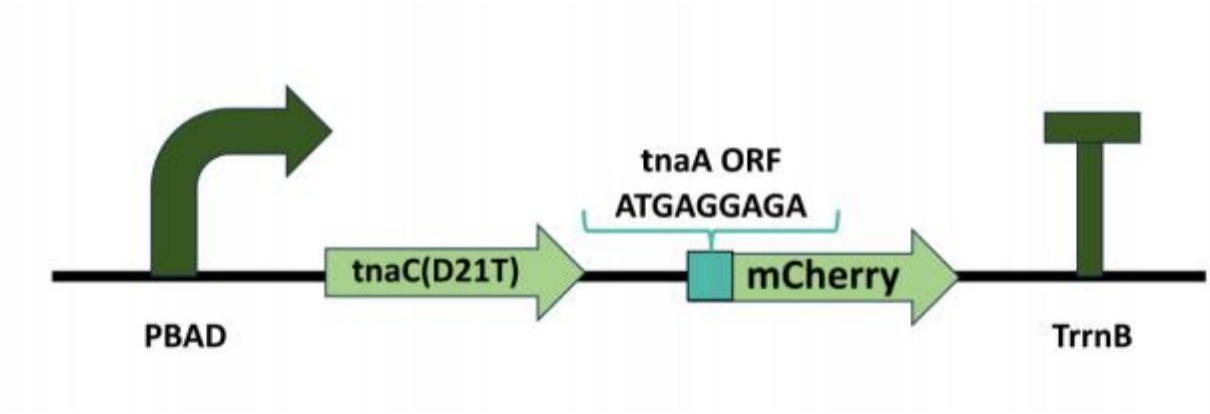
Take 100 ul of receptor cells mixed with 200 ng or more of the targeting fragment and incubate on ice for 10-30 min. Wash and dry the electrotransfer cup, and place it in an ultra clean table for UV sterilisation for 20 minutes. Subsequently, the sensory cells were quickly transferred to the bottom of the electrotransferring cup, the wall of the cup was wiped dry, and the cells were put into the electrotransferring instrument for electrotransferring. After electrotransformation, 1000 μL of 37℃ pre-warmed LB medium was added immediately, gently mixed, and transferred to a 1.5 mL centrifuge tube. After incubation in a shaker at 30°C and 150 rpm for 45-60 min, remove and spread on a selective plate containing Kana and Str double antibodies. One third of the 20 single colonies were selected for PCR verification (with the original Bw25113 as negative control)， The results showed that the band of the knockout strain was 1000bp and that of the original strain was 1800bpTo prove that the CysE gene was knocked out successfully。The results of the colony PCR are as follows：





(B) Testing Biosensor pLB1s-PBAD-tnaC(D21T)-mCherry

1、Plasmid Map



2、Transformation of the successfully ligated pLB1s-PBAD-tnaC(D21T)-mCherry plasmid into BW25113, with simultaneous transformation of the pLB1s-PBAD-tnaC-mCherry plasmid as a control.

Using the chemical transformation method, the ligation product was introduced into DH5α competent cells and plated on LB agar plates containing streptomycin resistance. The plates were incubated overnight at 37°C. Expected resistant single colonies appeared on the plates, and individual colonies were subsequently selected for colony PCR.

3、Strain Activation

Two-thirds of the single colonies from the plate were individually picked and inoculated into two tubes containing 5 ml of LB liquid medium, each supplemented with 5 μL of streptomycin.

The cultures were incubated at 37°C with shaking at 200 rpm for 12 hours.

4、Induction of Expression in M9 Medium with Tryptophan Concentration Gradients

M9 medium with gradient concentrations of tryptophan (0.01, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, and 0.50 g/L) was prepared.

50 μL of the cultured BW-pLB1s-PBAD-tnaC-mCherry and BW-pLB1s-PBAD-tnaC(D21T)-mCherry bacterial suspensions were separately added to 5 ml of the prepared M9 medium.

The cultures were incubated at 30°C with shaking at 200 rpm for 16 hours.

5、Microplate Reader Detection

The induced bacterial cultures were sequentially added to a 96-well plate, with 200 μL per well.

Based on the characteristics of the mCherry fluorescent protein, a fluorescence detection program

was set with an excitation wavelength of 552 nm and an emission wavelength of 600 nm. The OD value of the bacterial culture was measured at a wavelength of 600 nm.

The ratio of fluorescence intensity (RFU) to OD600 was calculated for subsequent data analysis.

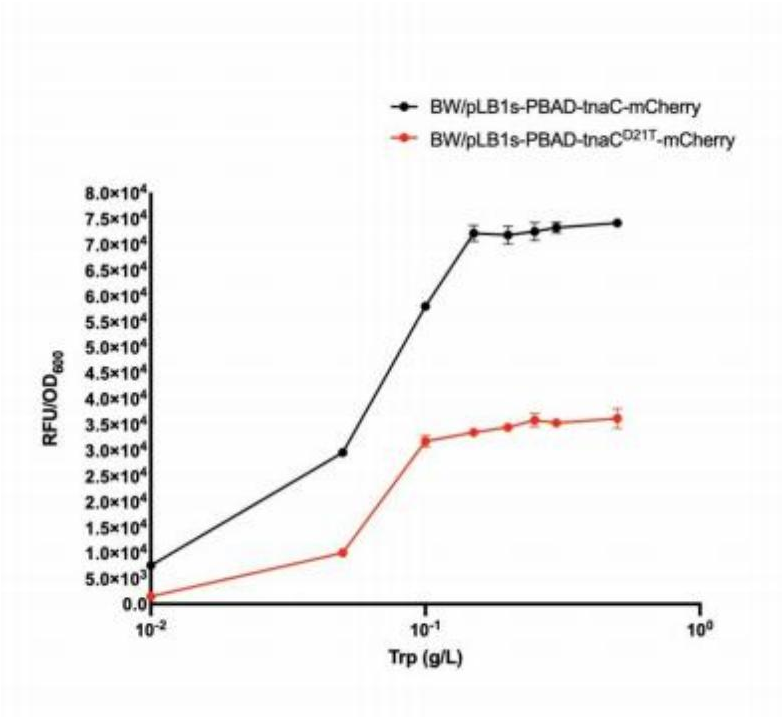
6、Data Analysis

The results for BW-pLB1s-PBAD-tnaC-mCherry are as follows:

Tryptophan(g/L)	RFU/OD600 1 组	RFU/OD6002 组	RFU/OD600 3 组
0.01	7427.1844660	7534.8928640	7603.5761010
0.05	29491.4816700	28878.9954300	30019.8895000
0.10	57174.4132500	58175.6756800	58323.9758800
0.15	72663.4335600	70312.1516200	73217.1964100
0.20	73590.2741900	71339.9602400	70242.3167800
0.25	71646.3525600	74389.5091300	71193.9991800
0.30	72442.9223700	74316.0593300	72696.9643800
0.50	73754.5933900	74352.0233600	/

The results for BW-pLB1s-PBAD-tnaC(D21T) -mCherry are as follows:

Tryptophan(g/L)	RFU/OD600 1 组	RFU/OD6002 组	RFU/OD600 3 组
0.01	1406.2500000	1524.9088700	1480.9590970
0.05	9902.6606100	10635.4060500	9357.8911790
0.10	31772.1088400	32645.0511900	30462.8766700
0.15	33602.7633900	33178.5480700	33330.2122300
0.20	34381.2814100	34763.7292500	33893.5912900
0.25	36576.6331700	34269.0678000	36441.7989400
0.30	36166.3479900	35029.3542100	34499.3026500
0.50	37434.7950700	34701.7543900	/

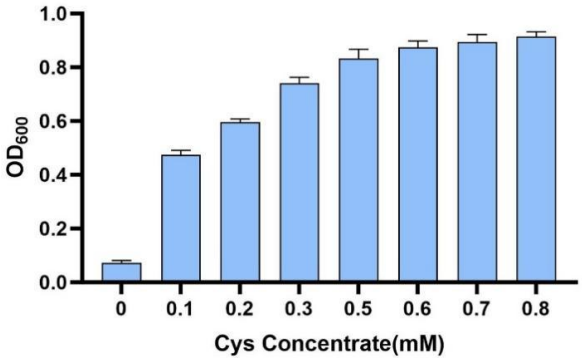


Based on the chart data analysis, the tryptophan response fold change of the mutated pLB1s-PBAD-tnaC(D21T)-mCherry biosensor is approximately 25-fold, which demonstrates superior performance compared to the 10-fold response observed in the control group (pLB1s-PBAD-tnaC-mCherry sensor) retested in this experiment.

Week4: 4.22-4.28:

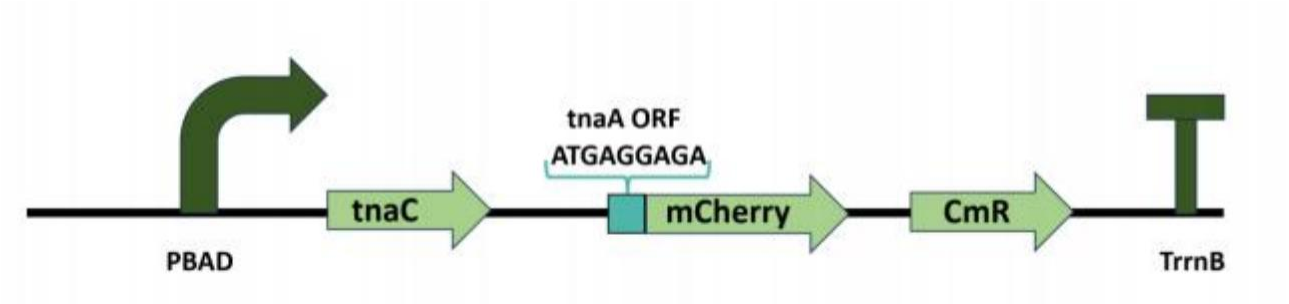
（A）Validation of CysE knockout

To verify the corresponding effects of different cysteine concentrations on the auxotrophic strain, we externally supplemented Bw25113 (a CysE-knockout strain) with varying concentrations of cysteine and monitored its growth status. As shown in the figure, the strain's growth improved with increasing cysteine concentration. Notably, the strain died without cysteine supplementation. These findings confirm that this strain is a cysteine auxotrophic mutant.



(C) Testing Biosensor pLB1s-PBAD-tnaC-mCherry-CmR

1、Plasmid Map



2、Transformation of the successfully ligated pLB1s-PBAD-tnaC-mCherry-CmR plasmid into BW25113

Using the chemical transformation method, the ligation product was introduced into DH5α competent cells and plated on LB agar plates containing streptomycin resistance. The plates were incubated overnight at 37°C. Expected resistant single colonies appeared on the plates, and individual colonies were subsequently selected for colony PCR.

3、Strain Activation

Two-thirds of the single colonies from the plate were picked and inoculated into 5 ml of LB liquid medium supplemented with 5 μL of streptomycin.

The cultures were incubated at 37°C with shaking at 200 rpm for 12 hours.

4、Induction of Expression in M9 Medium with Tryptophan Concentration Gradients

Three sets of M9 medium were prepared with tryptophan concentration gradients (0, 0.15, and 0.5 g/L) and chloramphenicol concentration gradients (0, 10, 20, 30, 40, 50, and 60 mg/L).

50 μL of the cultured BW-pLB1s-PBAD-tnaC-mCherry-CmR bacterial suspension was added to 5 ml of the prepared M9 medium.

The cultures were incubated at 30°C with shaking at 200 rpm for 16 hours.

5、Microplate Reader Detection

The induced bacterial cultures were sequentially added to a 96-well plate, with 200 μL per well.

The OD value of the bacterial culture was measured at a wavelength of 600 nm.

Data analysis was performed.

6、Data Analysis

When the exogenous tryptophan concentration was 0 g/L:

氯霉素(g/L)	OD600（1 组）	OD600（2 组）	OD600（3 组）
0	0.4272	0.5015	0.4831
10	0.2114	0.2827	0.2380
20	0.1026	0.1267	0.1354
30	0.0649	0.1053	0.0794
40	0.0584	0.0979	0.0626
50	0.0510	0.1019	0.0555
60	0.0550	0.0974	0.0515

When the exogenous tryptophan concentration was 0.15 g/L:

氯霉素(g/L)	OD600（1 组）	OD600（2 组）	OD600（3 组）
0	0.5297	0.4591	0.4344
10	0.3535	0.3354	0.3194
20	0.1768	0.1692	0.1597
30	0.1345	0.1152	0.1177
40	0.1154	0.0715	0.1084
50	0.1086	0.0624	0.0979
60	0.0976	0.0550	0.0791

When the exogenous tryptophan concentration was 0.5 g/L:

氯霉素(g/L)	OD600（1 组）	OD600（2 组）	OD600（3 组）
0	0.4438	0.4666	0.5043
10	0.2714	0.3101	0.3326
20	0.1433	0.1449	0.1321
30	0.1080	0.1127	0.1005
40	0.0625	0.1050	0.0701
50	0.0513	0.1070	0.0775
60	0.0530	0.1000	0.0636

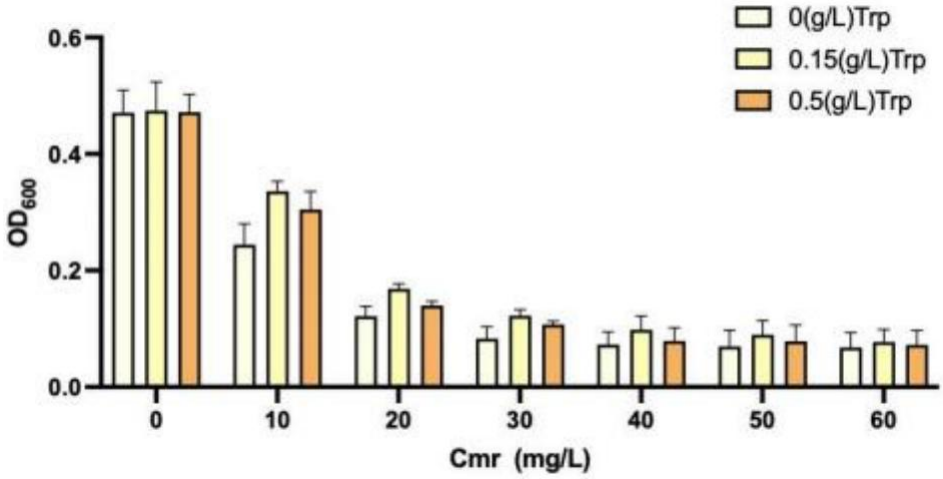


Chart analysis revealed that an exogenous chloramphenicol concentration of 10 mg/L inhibited bacterial growth. This indicates that 10 mg/L chloramphenicol can be used to screen for improved tryptophan-producing strains transformed with this biosensor. However, since the growth differences under varying tryptophan concentrations were minimal, the screening efficiency based on this method was suboptimal. To enhance screening efficiency, we plan to couple the mutant pLB1s-PBAD-tnaC(D21T)-mCherry with the chloramphenicol resistance gene (CmR), aiming to develop a novel biosensor that significantly amplifies OD value differences between different tryptophan concentrations upon chloramphenicol addition.