The experimental objective in March: To construct tryptophan biosensors/plasmids, knockout/mutate genes, and verify functions for screening high-tryptophan strains.

Week1: 3.1-3.7：

（A）Construction of pLB1s-VioABCDE and pSB1c-VioABCDE plasmids:

1. （1）Construction of pLB1s plasmids:

The target vector fragment was obtained by PCR. The PCR system is as follows.

PCR system (50μL)	
2×Mix	25μL
pLB1s-F	2μL
pLB1s-R	2μL
Template	20ng
DDW	20μL

PCR		
98℃	5min	
98℃	30s	×30
56℃	30s	
72℃	140s	
72℃	5min	
25℃	∞	

The PCR products were detected by agarose gel electrophoresis, and the correct target fragment length of 4173 bp was obtained. We obtained the correct target fragment and performed gel excision and recovery on the sample.

1. (2) Amplification of the vector pSB1c by PCR

The target carrier fragment was obtained by PCR, and the RCR system is as follows.

PCR system (50μL)	
2×Mix	25μL
pSB1c-F	2μL
pSB1c-R	2μL
Template	20ng
DDW	20μL

PCR		
98℃	5min	
98℃	30s	×30
56℃	30s	
72℃	140s	
72℃	5min	
25℃	∞	

The PCR products were detected by agarose gel electrophoresis, and the correct target fragment length of 4594 bp was obtained. We obtained the correct target fragment and performed gel recollection on the samples.

2.（1）By performing PCR amplification of the VioA fragment

The target fragment was obtained through PCR. The RCR system is as follows

PCR system (50μL)

2×Mix	25μL
VioA-F	2μL
VioA-R	2μL
template	20ng
DDW	20μL

98℃	5min	
98℃	30s	×30
52℃	30s	
72℃	40s	
72℃	5min	
25℃	∞	

PCR

The PCR products were detected by agarose gel electrophoresis, and the correct target fragment length of 1332 bp was obtained. We obtained the correct target fragment and performed gel recollection on the samples.

2. (2) Amplification of the VioB fragment by PCR

The target fragment was obtained through PCR. The RCR system is as follows.

PCR system (50μL)	
2×Mix	25μL
VioB-F	2μL
VioB-R	2μL
template	20ng
DDW	20μL

PCR		
98℃	5min	
98℃	30s	×30
52℃	30s	
72℃	95s	
72℃	5min	
25℃	∞	

The PCR products were detected by agarose gel electrophoresis, and the correct target fragment length of 3041 bp was obtained. We obtained the correct target fragment and performed gel recollection on the samples.

2. (3) Amplification of the VioC fragment by PCR

The target fragment was obtained through PCR. The RCR system is as follows.

PCR system (50μL)	
2×Mix	25μL
VioC-F	2μL
VioC-R	2μL
template	20ng
DDW	20μL

PCR		
98℃	5min	
98℃	30s	×30
52℃	30s	
72℃	40s	
72℃	5min	
25℃	∞	

The PCR products were detected by agarose gel electrophoresis, and the correct target fragment length of 1320 bp was obtained. We obtained the correct target fragment and performed gel recollection on the samples.

2. (4) Amplification of the VioD fragment by PCR

The target fragment was obtained through PCR. The RCR system is as follows.

PCR system (50μL)

2×Mix	25μL
VioD-F	2μL
VioD-R	2μL
template	20ng
DDW	20μL

PCR		
98℃	5min	
98℃	30s	×30
52℃	30s	
72℃	40s	
72℃	5min	
25℃	∞	

The PCR products were detected by agarose gel electrophoresis, and the correct target fragment length of 1144 bp as obtained. We obtained the correct target fragment and performed gel recollection on the samples.

2. (5) Amplification of the VioE fragment by PCR

The target fragment was obtained through PCR. The RCR system is as follows.

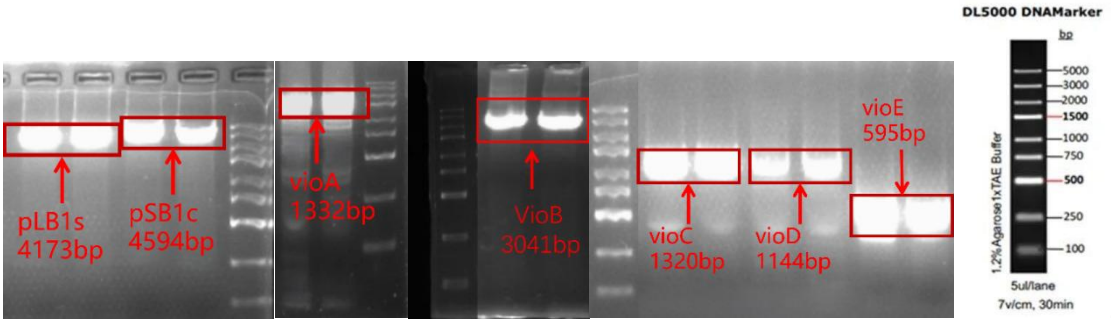
PCR system (50μL)	
2×Mix	25μL
VioE-F	2μL
VioE-R	2μL
template	20ng
DDW	20μL

PCR		
98℃	5min	
98℃	30s	×30
52℃	30s	
72℃	30s	
72℃	5min	
25℃	∞	

The PCR products were detected by agarose gel electrophoresis, and the correct target fragment length of 595 bp was obtained. We obtained the correct target fragment and performed gel recollection on the samples.

3. Gel Cutting and Recovery

The correct bands obtained were cut out and recovered. The PCR products were then subjected to agarose gel electrophoresis for detection. The results are as follows:



From left to right, they are pLB1s, pSB1c, VioA, VioB, VioC, VioD, VioE. Each fragment has 2 bands.

4. The six fragments were connected using the Gibsion assembly method.

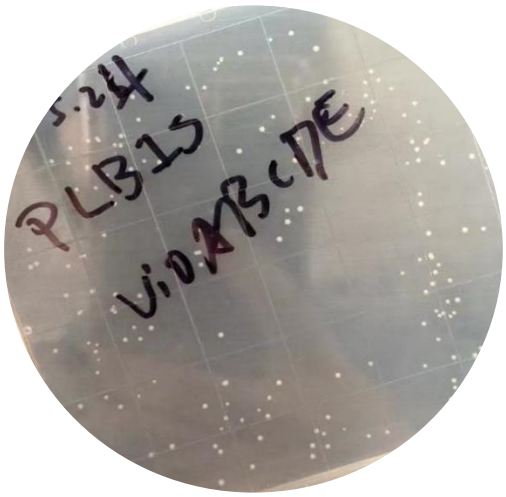
The specific operation is as follows:

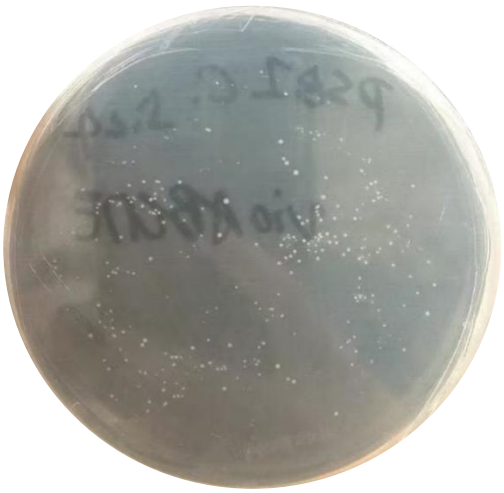
The pLB1s-VioABCDE and pSB1c-VioABCDE plasmids were obtained by using the Gibson (C116) connection. The lengths of pLB1, pSB1c, VioA, VioB, VioC, VioD, and VioE were 4173 bp, 4594 bp, 1332 bp, 3041 bp, 1320 bp, 1144 bp, and 595 bp, respectively.

pLB1s-VioABCDE Gibson system	
0.02×4173bp	ng
0.04×1332bp	ng
0.04×3041bp	ng
0.04×1320bp	ng
0.04×1144bp	ng
0.04×595bp	ng
2×c116 Mix	5μL
DDW	to10μL
pSB1c-VioABCDE Gibson system	
0.02×4594bp	ng
0.04×1332bp	ng
0.04×3041bp	ng
0.04×1320bp	ng
0.04×1144bp	ng
0.04×595bp	ng
2×c116 Mix	5μL
DDW	to10μL
Gibson	
50℃	45min
4℃	∞

5. Chemical Transformation:

Conjugated products were chemically transformed into DH5α competent cells, plated on LB plates with Spe and Chl resistance, and incubated at 37°C overnight.Resistant single colonies were picked for colony PCR.





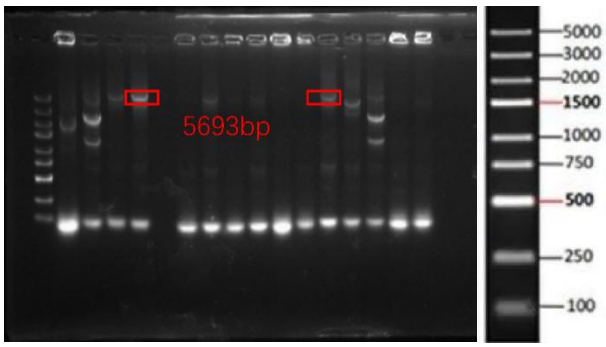
6. Colony PCR

After culturing at 37℃ for 12 hours, 10 colonies were selected from each plate. The PCR system for the colonies is as follows:

PCR system (10μL)		
×30	2×Hieff	25μL
	VioC-F	2μL
	VioE-R	2μL
	DDW	4.2μL

PCR		
98℃	5min	
98℃	30s	×30
57℃	30s	
72℃	170s	
72℃	5min	
25℃	∞	

The PCR products were subjected to agarose gel electrophoresis, and the results are as follows:

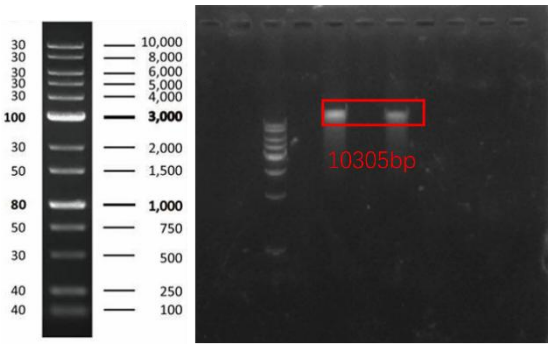


7. Monoclonal inoculation

Select the correct monoclonal and inoculate it into the liquid culture medium. Cultivate it at 37℃ for 12 hours.

8. Quality improvement and enzyme digestion verification

Single enzyme digestion verification was conducted using Bgl II. The enzyme digestion products were detected by agarose gel electrophoresis. The target bands were 10305bp and 1600bp. The result diagram is as follows:



9. The sequencing results of pLB1s-VioABCDE and pSB1c-VioABCDE are correct.

（B）Function verification of pLB1s-VioABCDE and pSB1c-VioABCDE plasmids (LB)

When the VioABCDE genes can all be expressed normally, tryptophan will be catalyzed by the protein expressed by the Vio gene to produce deoxypurpurin, and the colonies will appear purple. To verify the function of the constructed plasmids, the following steps are carried out:

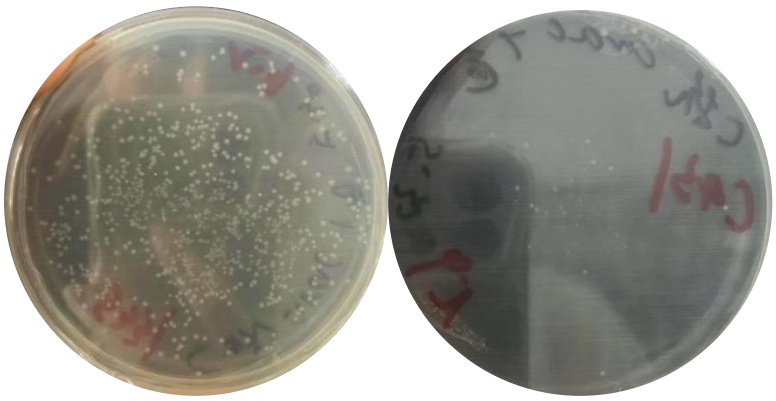
1. Prepare solid LB

Prepare a solid LB culture medium containing tryptophan and arabinose, as follows:

Solid Trp LB system	
LB(45℃, melted)	100mL
Arac	10μL
Trp	2g
antibiotic	100μL

2.Chemical Conversion

Using chemical transformation, the ligation products were transferred into BW25113 competent cells and spread onto tryptophan-arabinose LB plates containing Spe and Chl antibiotics. The plates were incubated at 37°C for 12 hours. The colony growth on the plates is as follows: no purple coloration was observed.



Considering that solid LB medium does not allow bacteria to fully access tryptophan, liquid LB medium was used instead. The preparation system is as follows:

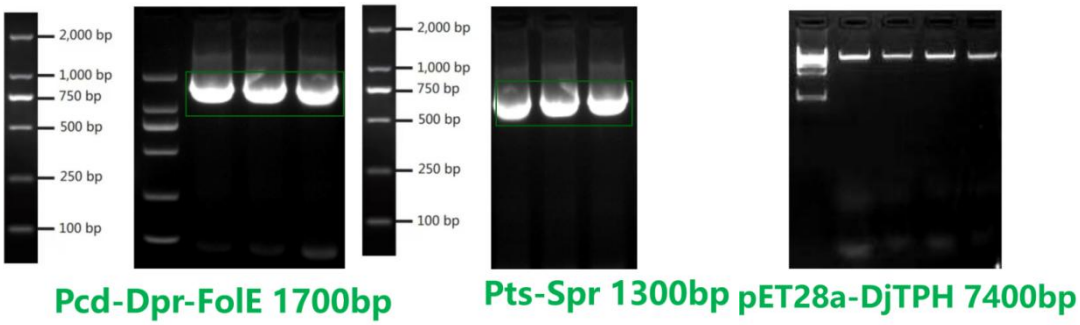
Liquid Trp LB system	
LB	5mL
Arac	1μL
Trp(10g/L)	10μL
Antibiotic	5μL
Bacterial liquid	50μL

Incubated at 37°C with shaking for 12 hours. Still no purple coloration appeared.

(C) pET-BH4 plasmid construction:

1. The vector was obtained by PCR amplification

In order to verify the functions of a series of genes involved in L-trp synthesis of 5-HTP, we constructed the relevant genes into pET28a vector. First, we amplified the target gene fragment by PCR and digested the vector with restriction enzymes to obtain the following results:



2.connection

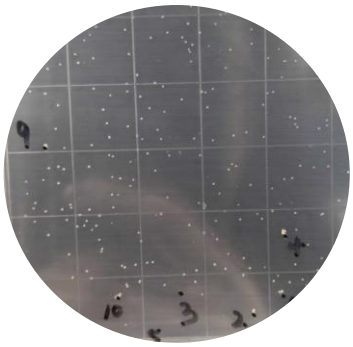
Connecting the three fragments using the Gibsion assembly method

Get the pET28a-BH4 plasmid

Gibson system	
pET28a-DjTPH	140ng
Pcd-Dpr-FolE	34ng
Pts-Spr	26ng
2×c116 Mix	5ul
DDW	to10ul

3.Chemical transformation

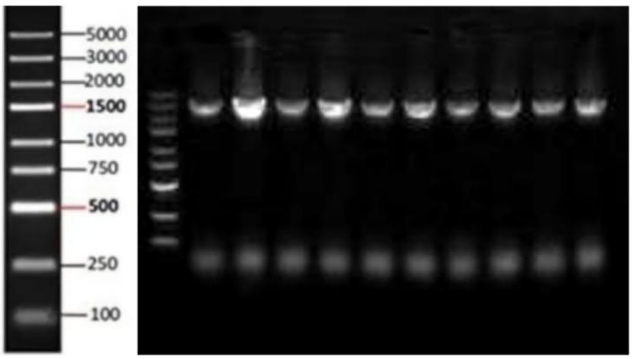
Using the chemical transformation method, the conjugation product was transferred into DH5α competent cells and plated on LB agar plates containing Amp resistance. The plates were incubated overnight at 37°C. When expected antibiotic-resistant single colonies emerged, 10 colonies were selected for PCR analysis. The bacterial plasmid system is shown in the figure.:



PCR system (10μL)	
2×Hieff	5ul
JP-F	0.4ul
JP-R	0.4ul
DDW	4.2ul

PCR		
98℃	5min	
98℃	30s	×30
57℃	30s	
72℃	1min30s	
72℃	5min	
4℃	∞	

The results of colony PCR were as follows, with a positive rate of 100%:

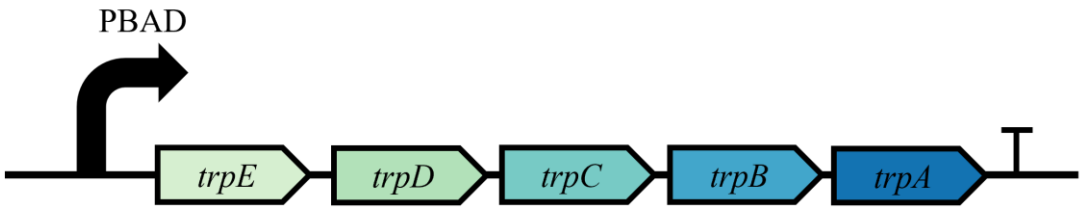


4.Extract plasmid, send for sequencing

The plasmid was extracted and sequenced. pET-BH4 plasmid sequencing results were correct.

(D) Construction of the overexpression plasmid pYB1a-trpEDCBA

1. Constructing Plasmid Maps



PCR to obtain the target gene and vector

Using the E. coli K12 genome as the template for trpEDCBA and pYB1a-eGFP as the template for the vector pYB1a, the target genes and vectors were obtained through PCR experiments.

PCR system:

trpED reaction system（50μL）	
BW25113	1μL
trpED-F	2μL
trpED-R	2μL
2×HF Mix	25μL
ddw	20μL

trpCBA reaction system（50μL）	
BW25113	1μL
trpCBA-F	2μL
trpCBA-R	2μL
2×HF Mix	25μL
ddw	20μL

pYB1a reaction system（50μL）	
pYB1a-eGFP	1μL
pYB1a-F	2μL
pYB1a -R	2μL
2×HF Mix	25μL
ddw	20μL

2.PCR Program Settings

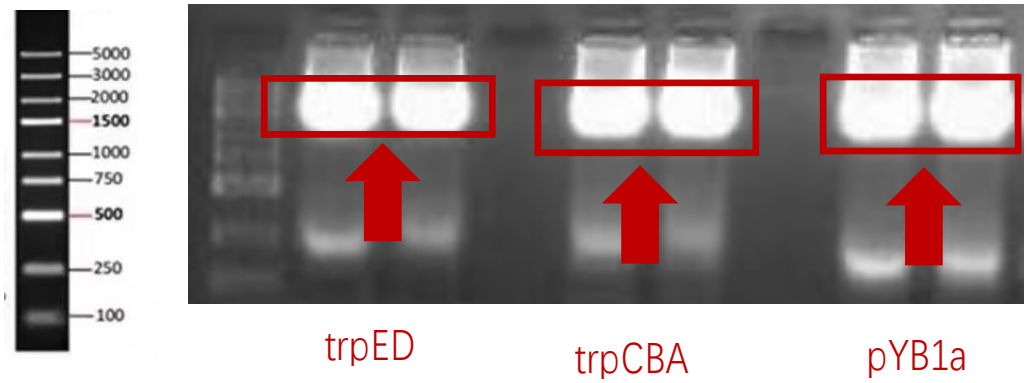
trp-ED reaction system			
98℃	5min		× 30
98℃	30s		
58℃	30s		
72℃	120s		
72℃	5min		
25℃		∞	

trp-CBA reaction system			
98℃	5min		× 30
98℃	30s		
59℃	30s		
72℃	138s		
72℃	5min		
25℃		∞	

pYB1a reaction system			
98℃	5min		× 30
98℃	30s		
57℃	30s		
72℃	108s		
72℃	5min		
25℃		∞	

3.Gel-cutting and recycling of trpED and trpCBA fragments

The correct band was excised from the gel and recovered. The PCR products were analyzed by agarose gel electrophoresis, yielding the following results:



4.Perform Dpn I digestion on the pYB1a fragment and purify the resulting product.

DpnIDigestive Reaction System:

DpnIDigestive Reaction System（50μL）	
pYB1a	5μL
DpnIRestriction enzyme	1μL
rcutsmart Buffer solution	5μL
ddw	39μL

DpnIDigestion Program Settings:

DpnIDigestion Program Settings	
37℃	4h
25℃	∞

Purify the digested products.

5.Gibson Connection

Connectivity System：

Ginson Connectivity System（10μL）	
pYB1a	1.5μL
trpED	0.4μL
trpCBA	0.5μL
2×CE Mix	5μL
ddw	2.6μL

Connection Program：

Gibson Connection Program Settings	
55℃	30min
4℃	∞

6.Chemical Conversion

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

7.Colony PCR

Colony PCR System：

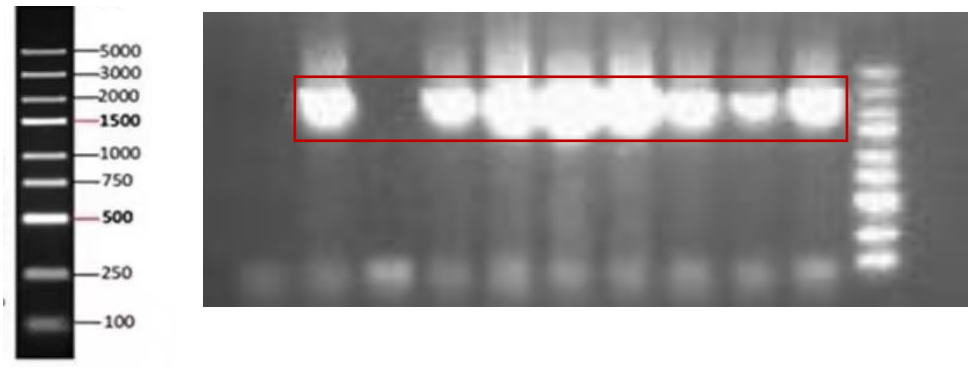
Colony PCR Reaction System（10μL）	
bacterial solution	1μL
trpEDCBA-F	0.4μL
trpEDCBA-R	0.4μL
Green Mix	5μL
ddw	3.2μL

Colony PCR Protocol Settings：

pYB1a Reaction Procedure		
98℃	5min	
98℃	30s	× 30
57℃	30s	
72℃	60s	
72℃	5min	
25℃	∞	

Perform agarose gel electrophoresis on the colony PCR results.

The results are as follows:



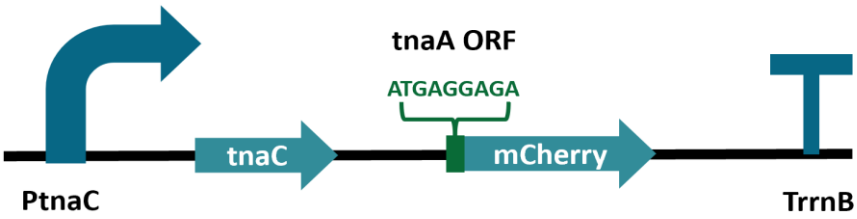
The colony PCR band size matched the expected 975 bp, with a positivity rate of 80%.

8.Plasmid extraction and sequencing service

The sequencing results are correct.

(E) Construction of the biosensor pYB1a-tnaC-mCherry

1. Constructing Plasmid Maps



2. PCR to obtain the target gene and vector

Using the E. coli K12 genome as the template for tnaC, puam-O as the template for mCherry, and pYB1a-eGFP as the template for the vector pYB1a. The target genes and vectors were obtained through PCR experiments.

PCR system:

tnaC reaction system (50μL)	
BW25113	1μL
tnaC-F	2μL
tnaC-R	2μL
2×HF Mix	25μL
ddw	20μL

mCherry reaction system (50μL)	
puam-O	1μL
mCherry-F	2μL
mCherry-R	2μL
2×HF Mix	25μL
ddw	20μL

pYB1a reaction system (50μL)	
pYB1a-eGFP	1μL
pYB1a-F	2μL
pYB1a -R	2μL
2×HF Mix	25μL
ddw	20μL

PCR Program Settings:

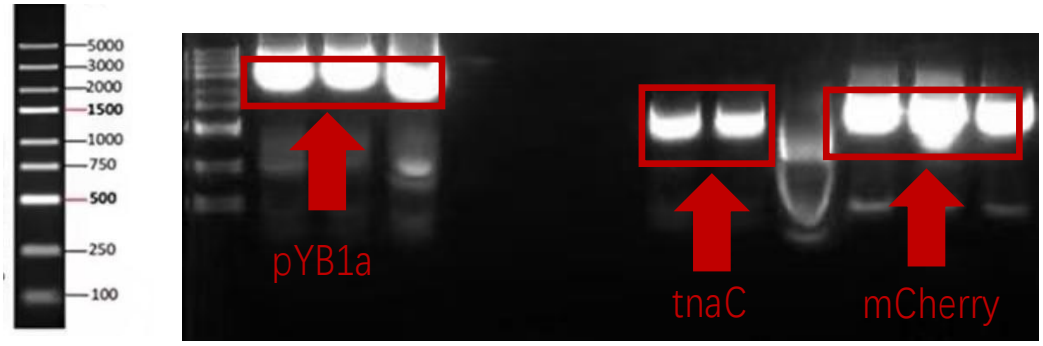
tnaC Reaction Procedure			× 30
98℃	5min		
98℃	30s		
57℃	30s		
72℃	30s		
72℃	5min		
25℃		∞	

mCherry Reaction Procedure			× 30
98℃	5min		
98℃	30s		
57℃	30s		
72℃	30s		
72℃	5min		
25℃		∞	

pYB1a Reaction Procedure			× 30
98℃	5min		
98℃	30s		
54℃	30s		
72℃	108s		
72℃	5min		
25℃		∞	

3. Gel-cutting and recovery of tnaC, mCherry, and pYB1a fragments

The correct band was excised from the gel and recovered. The PCR products were analyzed by agarose gel electrophoresis, yielding the following results:



4. Gibson Connection

Connectivity System:

Ginson Connectivity System（10μL）	
pYB1a	1μL
tnaC	1μL
mCherry	1μL
2×CE Mix	5μL
ddw	2μL

Connection Program:

Gibson Connection Program Settings	
50℃	15min
4℃	∞

5. Transform the ligation product into DH5

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

6. Colony PCR

Colony PCR System:

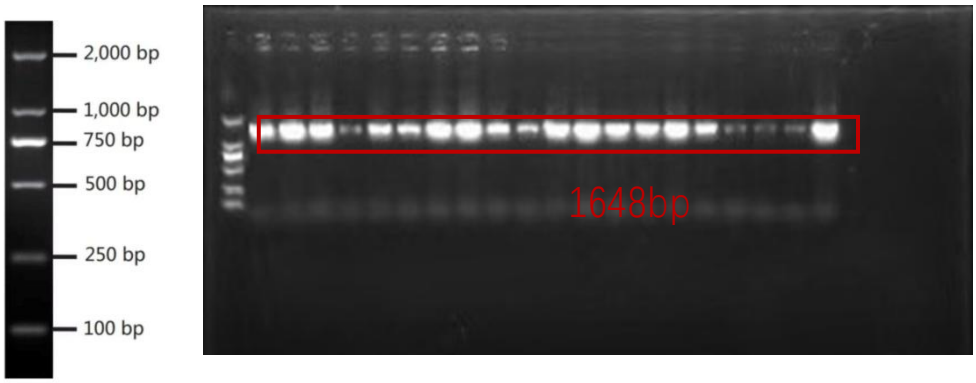
Colony PCR Reaction System (10μL)	
bacterial solution	1μL
tnaC-mCherry-F	0.4μL
tnaC-mCherry -R	0.4μL
Green Mix enzyme	5μL
ddw	3.2μL

Colony PCR Protocol Settings:

Colony PCR Reaction Protocol		
98℃	5min	
98℃	30s	× 30
57℃	30s	
72℃	60s	
72℃	5min	
25℃	∞	

Perform agarose gel electrophoresis on the colony PCR results.

The results are as follows:



The colony PCR band size was as expected, with a 100% positive rate.

7. Extract recombinant plasmids and send them for sequencing.

Sequencing results successful.

Week2: 3.8-3.14

Attempt to validate plasmid function through whole-cell catalysis.

(A) Functional Verification of pLB1s-VioABCDE and pSB1c-VioABCDE Plasmids (Whole-Cell Catalysis)

1. Induction

Add arabinose and induce for 18 hours. The induction system is as follows:

system	
ZY	4.8mL
50×M	100μL
50×5052	100μL
1M $MgSO_4$	10μL
1000×	10μL
arac	50μL
Bacterial liquid	50μL
Antibiotic	5μL

After induction, centrifuge for enrichment. Add 200 μL of Tris-HCl-tryptophan solution, incubate at 30°C for 12 hours, centrifuge, yet no color change occurred.

(B) Verify the expression status of the VioABCDE genes

Suspecting that a gene in VioABCDE may not be expressed, SDS-PAGE was used to verify its expression status.

1. Chemical Conversion

Using chemical transformation, plasmids containing only a single gene were individually transformed into BL21 competent cells. The cells were then spread onto LB plates containing kanamycin resistance and incubated at 37°C for 12 hours. A single colony was picked and inoculated into ZY liquid medium, which was shaken for 12 hours.

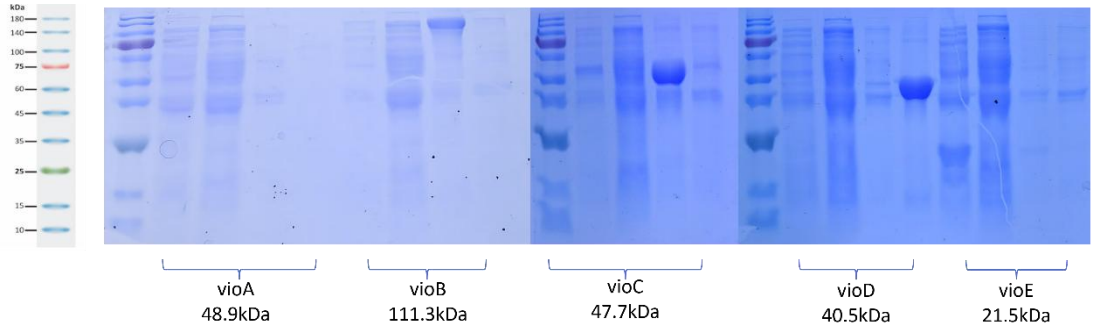
2. Induction

Add IPTG and induce for 18 hours, with a control group without IPTG. The induction system is as follows:

system	
ZY	4.8mL
50×M	100μL
50×5052	100μL
1M $MgSO_4$	10μL
1000×	10μL
IPTG	50μL
Bacterial liquid	50μL
Antibiotic	5μL

3 .SDS-PAGE electrophoresis validation

After sonication of the samples to prepare protein samples, SDS-PAGE electrophoresis was performed for validation. The results are as follows:



For E. coli, VioABCDE is an exogenous gene with suboptimal expression. Consequently, we no longer rely on this sensor and have instead shifted our focus to constructing and functionally validating a tryptophan sensor.

(C) Protein validation BH4 CrTDC SgAANAT AtCOMT exogenous gene

1.Pick monoclonal inoculation and Induction

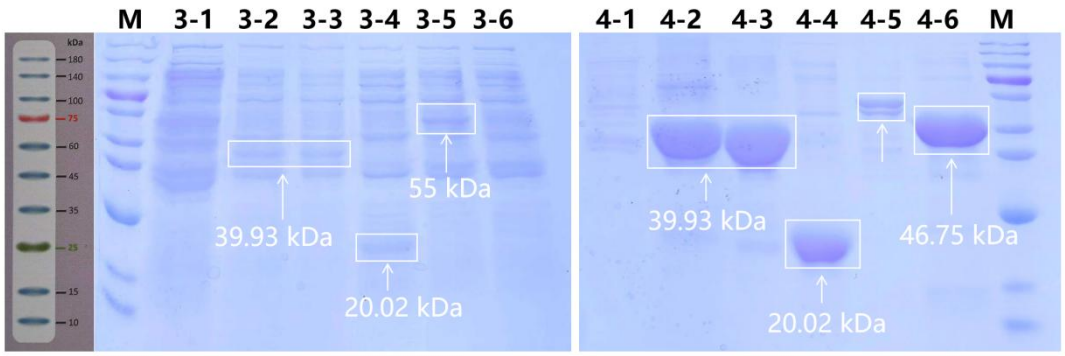
The constructed plasmids pET28a-BH4, pET28a-CrTDC, pET28a-SgAANAT, and pET28a-AtCOMT were transformed into BL21(DE3) bacterial cells. After selecting antibiotic-resistant single colonies from the plates, the bacterial cultures were inoculated and incubated at 37°C°C for 12 hours. Subsequently, all colonies were induced with 25°CM antibiotic at 37°C°C for 24 hours using the following induction protocol:

Induction system	
ZY	4.8ml
50×M	100μl
5052	100μl
IPTG	5μl
$MgSO_4$	10μl
1000×	10μl

Bacterial liquid	50μl
Antibiotic	5μl

2.SDS-PAGE detection

Protein detection of the induced strains was as follows:



The results showed protein expression in both supernatant and precipitation, but further verification is needed to confirm whether the gene functions properly

(D) Establishment of an HPLC Method for the Determination of Tryptophan

1. Preparations

To determine the yield of the product tryptophan, we first identified an HPLC method for tryptophan detection by reviewing the literature.

1. Stationary phase: Agilent C18 column (250 mm × 4.6 mm, 5 μm, Agilent).
2. Mobile phase: 0.3 g/L $KHPO_4$ (aqueous solution) mixed with methanol at a 9:1 (volume ratio).
3. UV detection wavelength: 278 nm.
4. Injection volume: 10 μL; flow rate: 1.0 mL/min; column temperature: 39°C.
5. Product retention time: Approximately 25 minutes.
6. Standard solution preparation: Prepare tryptophan solutions at concentrations of 1 μM/L, 2 μM/L, 3 μM/L, 4 μM/L, and 5 μM/L, then filter through a membrane.

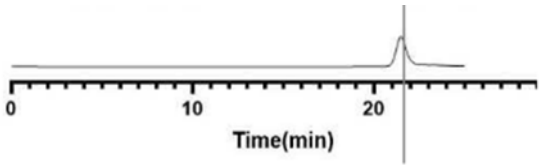
2. Experimental Procedure

1.Flush the tubing to remove air bubbles at a flow rate of 5.0 mL/min.

2. Turn on the infusion pump and allow the mobile phase to flow through the column at 0.7 mL/min until the baseline stabilizes.

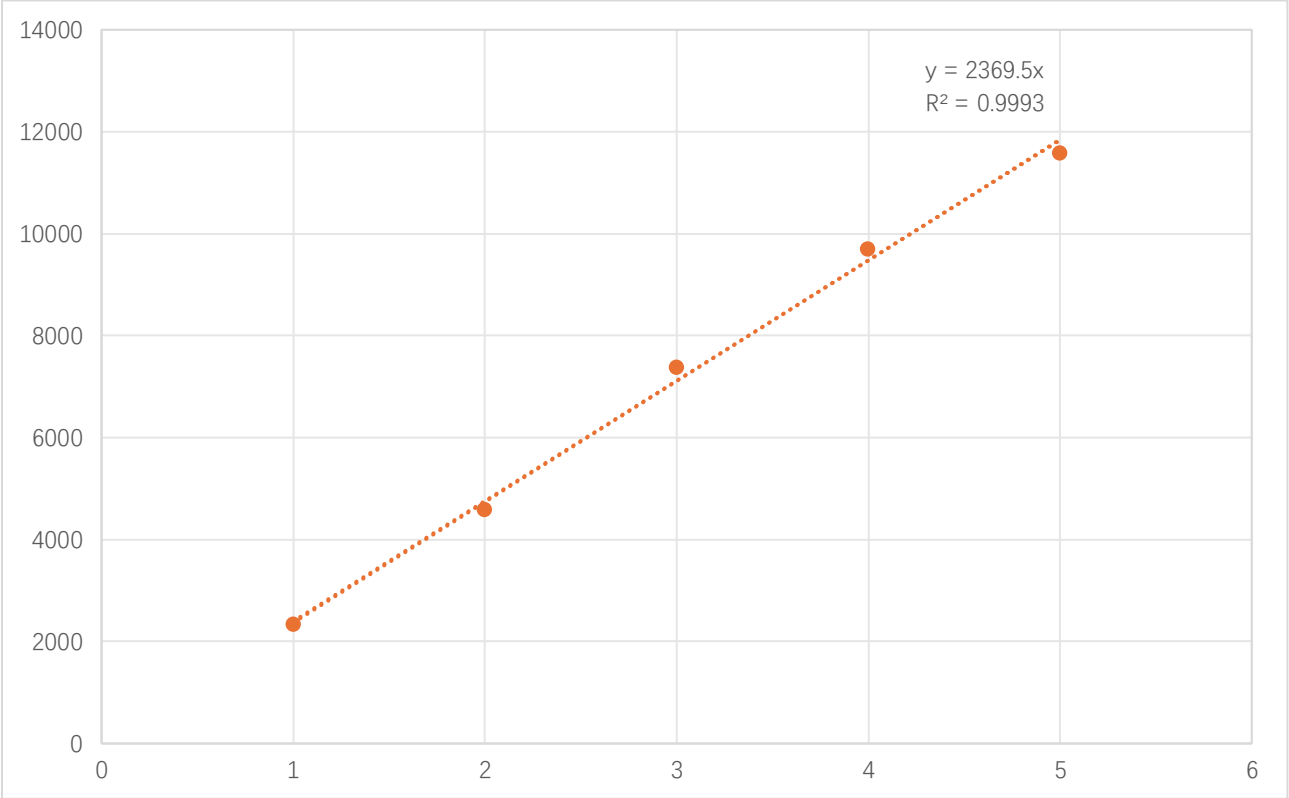
3. Inject standard solutions of varying concentrations into the HPLC system sequentially at a flow rate of 1.0 mL/min and an injection volume of 10 μL.

4. Obtain chromatograms showing peak elution at approximately 25 minutes.



3. Standard Curve Plotting

1. Plot a standard curve with tryptophan standard solution concentration on the x-axis and corresponding peak area on the y-axis.
2. Perform linear regression analysis on the standard curve to obtain the regression equation and correlation coefficient.



Tryptophan yield = Peak area / 2369.5

(E) Functional testing of the overexpressed plasmid pYB1a-trpEDCBA

1.Transform the plasmid into BW25113 competent cells.

Using chemical transformation, the ligation products were transferred into BW25113 competent cells and spread onto LB plates containing Amp resistance. The plates were incubated overnight at 37°C. The expected resistant single colonies grew on the plates, and subsequent single colonies were selected for colony PCR.

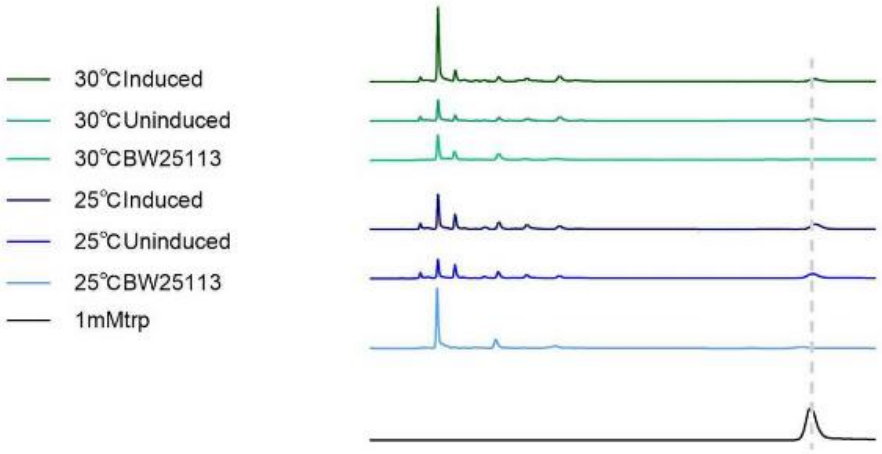
2.Induction, whole-cell catalysis

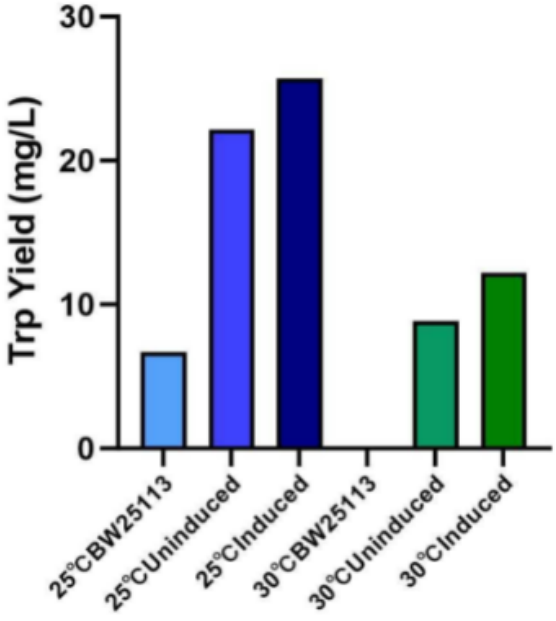
Transfer to ZY5052 autoinductive medium and induce at 25°C/30°C for 18 hours. After 18 hours, remove and place on ice for UV spectrophotometric measurement. Take a volume of culture corresponding to 600 OD, resuspend in M9 medium, and incubate at 30°C for 12 hours.

ZY5052 Reaction system（5mL）	
ZY medium	4.8mL
50×M	100μL
50×5052	100μL
1000×trace elements	10μL
1MgSO4	10μL
bacterial solution	50μL
Antibiotics	5μL
arac	50μL

3.HPLC analysis

1. Centrifuge the whole-cell catalytic products, collect the supernatant, and filter it through a membrane.
2. Determine the tryptophan content in the whole-cell catalytic products under identical conditions as the standard curve.
3. Results are as follows:



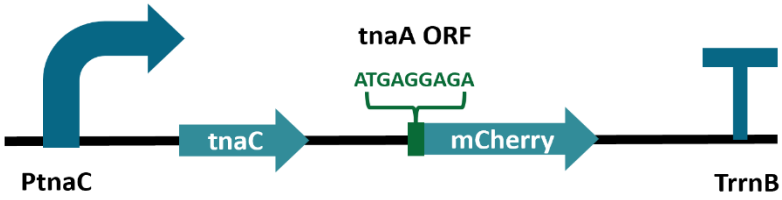


4.Results Analysis

The liquid phase results indicate that the tryptophan yield in strains transformed with the overexpression plasmid and subjected to induction showed a significant increase, demonstrating that overexpression of the trpEDCBA genes enhances tryptophan production. Furthermore, 25°C is the more suitable temperature for inducing tryptophan production.

(F) Testing the pYB1a-tnaC-mCherry biosensor

1.Plasmid Atlas



2.Transform the successfully ligated pYB1a-tnaC-mCherry plasmid into BW25113.

Using chemical transformation, the ligation products were transferred into BW25113 competent cells and spread onto LB plates containing Amp resistance. The plates were incubated overnight at 37°C. The expected resistant single colonies grew on the plates, and subsequent single colonies were selected for colony PCR.

3.Activated Strain

Transfer two-thirds of a single colony from the plate to 5 mL of LB liquid medium, and add 5 μL of ampicillin.

Incubate at 37°C with shaking at 200 rpm for 12 hours.

4.Induction of Expression by Tryptophan Gradient Concentrations in M9 Medium

Take the BW-pYB1a-tnaC-mCherry bacterial culture for induction and detect using a microplate reader. Finally, obtain the ratio of fluorescence intensity (RFU) to OD600.

5.Data Analysis

Tryptophan(g/L)	RFU/OD_{600}
BW25113	1648.355493
0	18351.11083
0.025	23673.10961
0.05	25041.66409
0.1	26028.44856
0.2	27176.67004
0.5	33695.46673
0.8	28534.83702
1.0	34115.89808

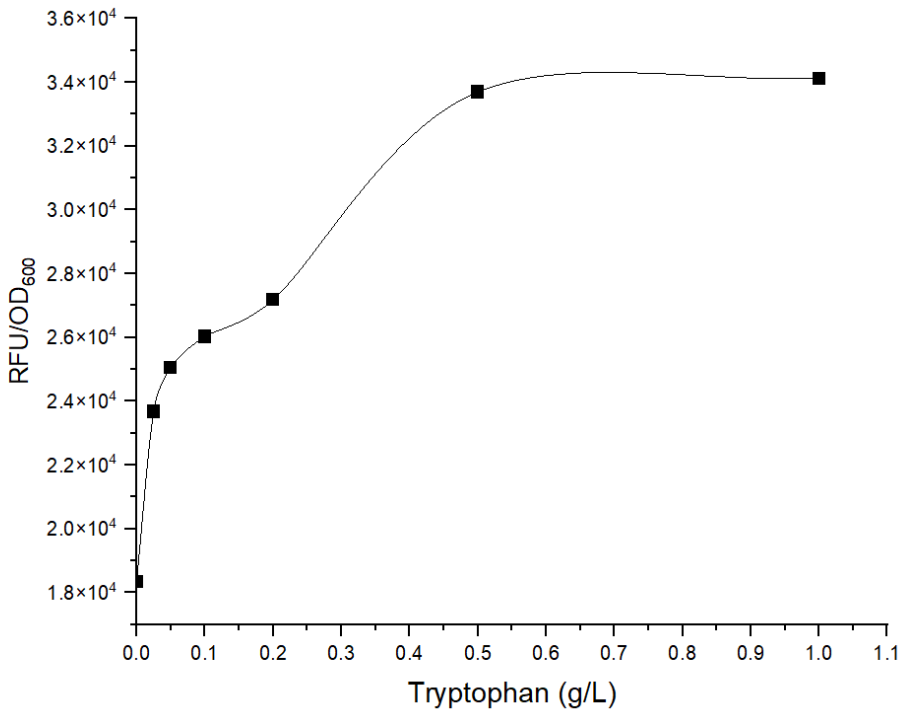
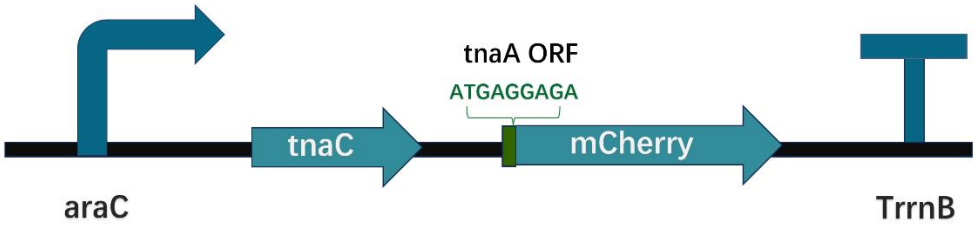


Image analysis indicates a sharp increase in RFU/OD600 values within the 0.0–0.05 g/L tryptophan concentration range. The pYB1a-tnaC-mCherry biosensor exhibits an excessively low tryptophan response threshold, rendering it unusable. This phenomenon is inferred to result from gene leakage, potentially caused by excessively high plasmid copy numbers or leakage from the tnaC promoter. Therefore, the next step involves replacing the promoter and adjusting the plasmid copy number to test their effectiveness.

(G) Construction of the pSB1c-tnaC-mCherry Biosensor

1.Constructin Plasmid Maps:



2.Obtain the target gene and vector

Amplify the tnaC-mCherry fragment via PCR using pYB1a-tnaC-mCherry as the template; Obtain the vector fragment by digesting pSB1c-eGFP with restriction enzymes.

PCR system：

tnaC-mCherry reaction system（50μL）	
pSB1c-eGFP	1μL
pSB1c-F	2μL
pSB1c -R	2μL
2×HF Mix	25μL
ddw	20μL

PCRProgram Settings：

tnaC-mCherry reaction system		
98℃	5min	
98℃	30s	× 30
55℃	30s	
72℃	33s	
72℃	5min	
25℃	∞	

pSB1c Enzyme Digestion System：

Enzyme digestion system（50μL）	
pSB1c-eGFP	13μL
rcutsmart	5μL
SpeI	1μL
Kpn1	1μL
ddw	30μL

pSB1c Restriction Enzyme Digestion Protocol：

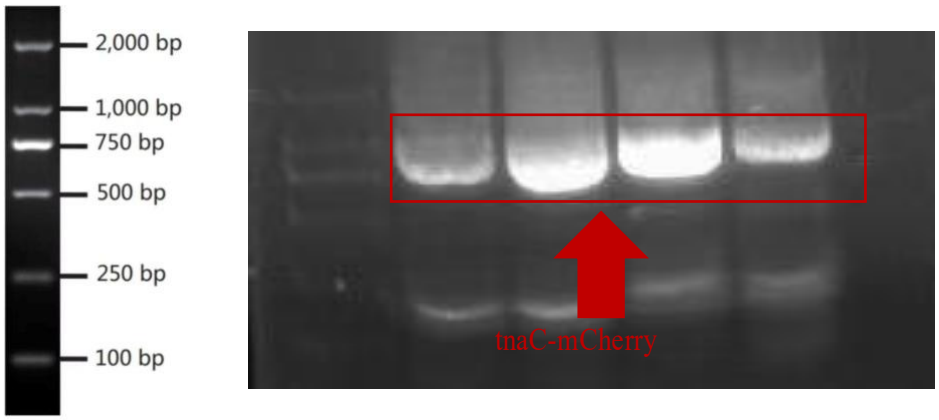
pSB1cRestriction Enzyme Digestion Protocol	
37℃	4.5h
25℃	∞

3.Gel-Cutting and Gene Fragment Recovery

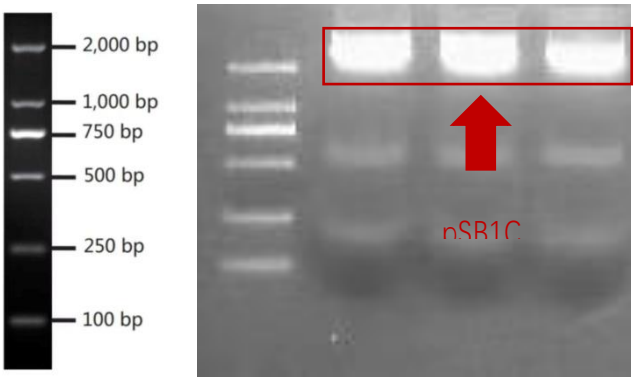
Perform agarose gel electrophoresis on the target gene and recover the fragment by cutting the gel.

The results are as follows:

tnac-mCherry Electrophoresis diagram：



pSB1c Enzyme digestion gel electrophoresis：



4.Gibson Connection

Connectivity System：

Ginson Connectivity System（10μL）	
pSB1c	1μL
tnaC	1μL
mCherry	1μL
2×CE Mix	5μL
ddw	2μL

Connection Program：

Gibson Connection Program Settings	
50℃	15min
4℃	∞

5.Transform the ligation product into DH5

Using chemical transformation, the ligation products were transferred into BW25113 competent cells and spread onto LB plates containing Amp resistance. The plates were incubated overnight at 37°C. The expected resistant single colonies grew on the plates, and subsequent single colonies were selected for colony PCR.

6.Colony PCR

Colony PCR System：

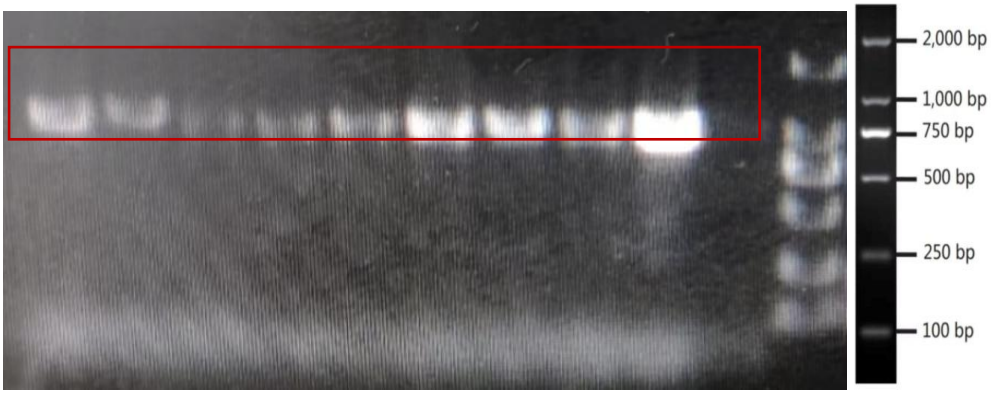
Colony PCR Reaction System（10μL）	
bacterial solution	1μL
pSB1c-F	0.4μL
pSB1c-R	0.4μL
Green Mix enzyme	5μL
ddw	3.2μL

Colony PCR Protocol Settings：

Colony PCR Reaction Protocol		
98℃	5min	
98℃	30s	× 30
58℃	30s	
72℃	18s	
72℃	5min	
25℃	∞	

Perform agarose gel electrophoresis on the colony PCR results.

The results are as follows:



The colony PCR band size was as expected, with a 90% positive rate.

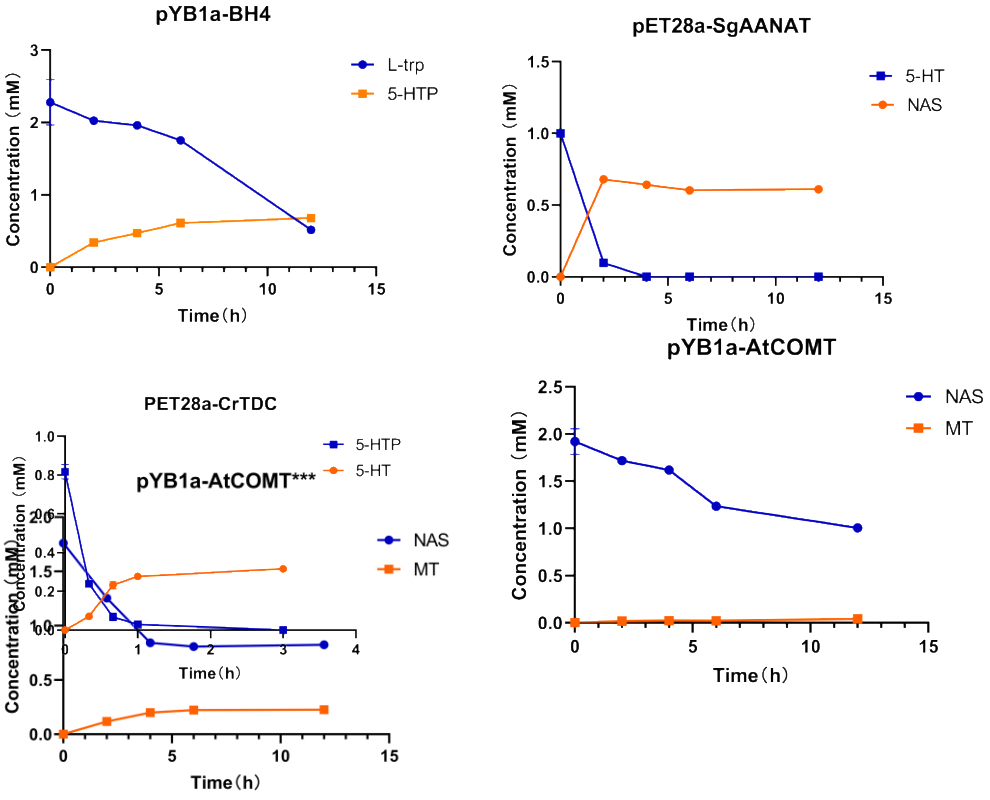
7.Extract recombinant plasmid

Plasmid extraction and sequencing. Sequencing results confirm successful construction of pSB1c-tnaC-mCherry. This construct can be transformed into BW25113 for induced expression and efficacy testing.

Week3 : 3.15-3.21

(A) BH4, CrTDC, SgAANAT, AtCOMT single plasmid functional validation

We measured the activity of each enzyme in the pathway. From L-trp to MT



(B) CRISPR-Cas9-mediated knockout of the trpR gene

1、Preparation of electrocompetent cells

Electrocompetent cells of BW25113 harboring the pEc-Cas9 plasmid were prepared and immediately stored in a -80°C freezer.

2、Preparation of the targeting fragment

Fragments of 500 bp each from the upstream and downstream regions of the trpR gene, designated as D500 and U500 respectively, were obtained by PCR. Meanwhile, the Donor vector was also amplified via PCR. The PCR system and procedure are as follows:

The U500 reaction system（50μL）	
Bacterial culture (broth)	1μL
trpR-U500-F	2μL
trpR-U500-R	2μL
2×HF Mix	25μL
ddw	20μL

The U500 reaction program		
98℃	5min	
98℃	30s	× 30
56℃	30s	
72℃	30s	
72℃	5min	
25℃	∞	

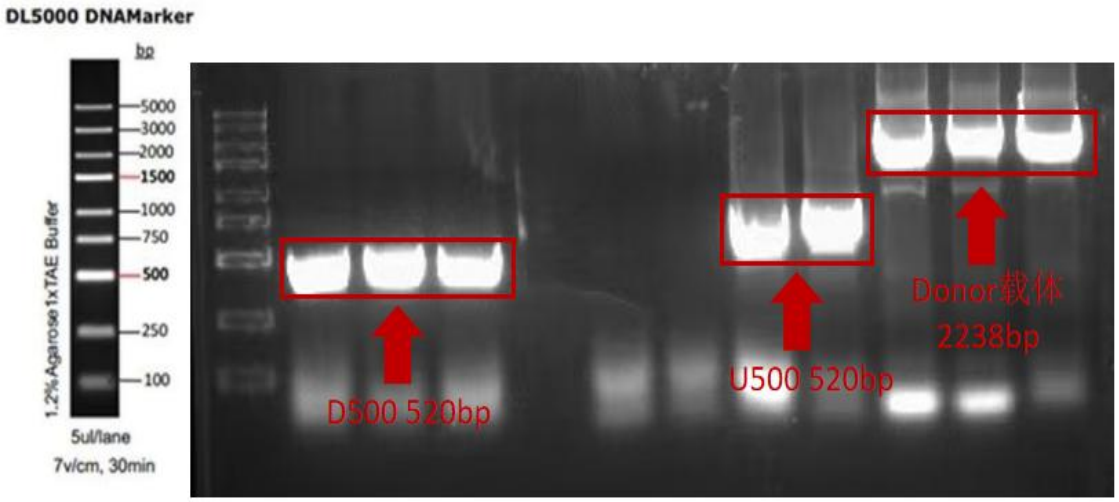
The D500 reaction system（50μL）	
Bacterial culture (broth)	1μL
trpR-D500-F	2μL
trpR-D500-R	2μL
2×HF Mix	25μL
ddw	20μL

The D500 reaction program		
98℃	5min	
98℃	30s	× 30
56℃	30s	
72℃	30s	
72℃	5min	
25℃	∞	

The Donor reaction system（50μL）	
The Donor plasmid	1μL
trpR-Do-F	2μL
trpR-Do- R	2μL
2×HF Mix	25μL
ddw	20μL

The Donor reaction program		
98℃	5min	
98℃	30s	× 30
55℃	30s	
72℃	1min9s	
72℃	5min	
25℃	∞	

Subsequently, the PCR products were subjected to agarose gel electrophoresis, followed by gel extraction and purification.The results of agarose gel electrophoresis are as follows:



Then, we performed Gibson assembly on several fragments.
The assembly system is as follows:

Gibson assembly system （10μL）	
The Donor vector	1.4μL
U500	1.2μL
D500	1.4μL
2×CE Mix	5μL
ddw	1μL

The Gibson assembly program is as follows：

Gibson assembly program settings	
55℃	30min
4℃	∞

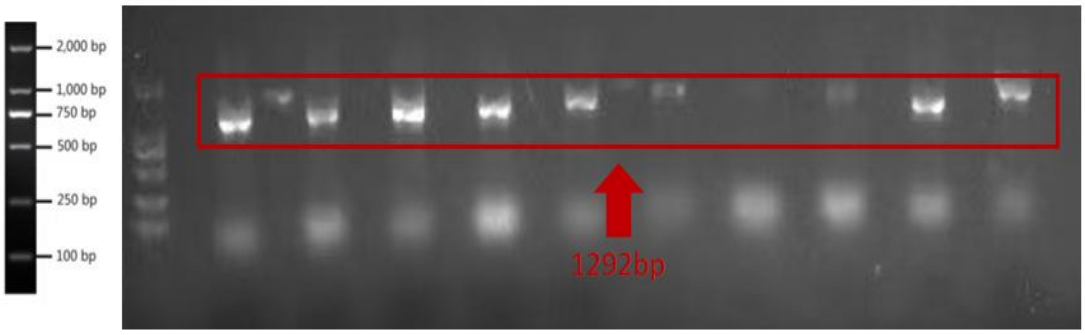
We performed chemical transformation by introducing the ligation products into DH5α competent cells, followed by plating onto LB agar plates supplemented with streptomycin (Str) selection marker. The plates were then incubated overnight at 37°C in a humidified incubator. Expected streptomycin-resistant single colonies emerged on the plates. Subsequently, individual colonies were picked for colony PCR screening to verify positive clones. The colony PCR reaction mixture is as follows:

The reaction mixture for colony PCR （10μL）	
Picking single colonies	
DO-trpR-JP-F	0.4μL
DO-trpR-JP -R	0.4μL
Green Mix enzyme	5μL
ddw	4.2μL

The reaction program for colony PCR is as follows:

The reaction program for colony PCR		
98℃	5min	× 30
98℃	30s	
57℃	30s	
72℃	20s	
72℃	5min	
25℃	∞	

The colony PCR results were analyzed by agarose gel electrophoresis. The results are as follows:



Subsequently, the samples were sent for sequencing, and the results were confirmed to be correct.

Reaction mixture for obtaining linear D500 and U500 fragment via PCR:

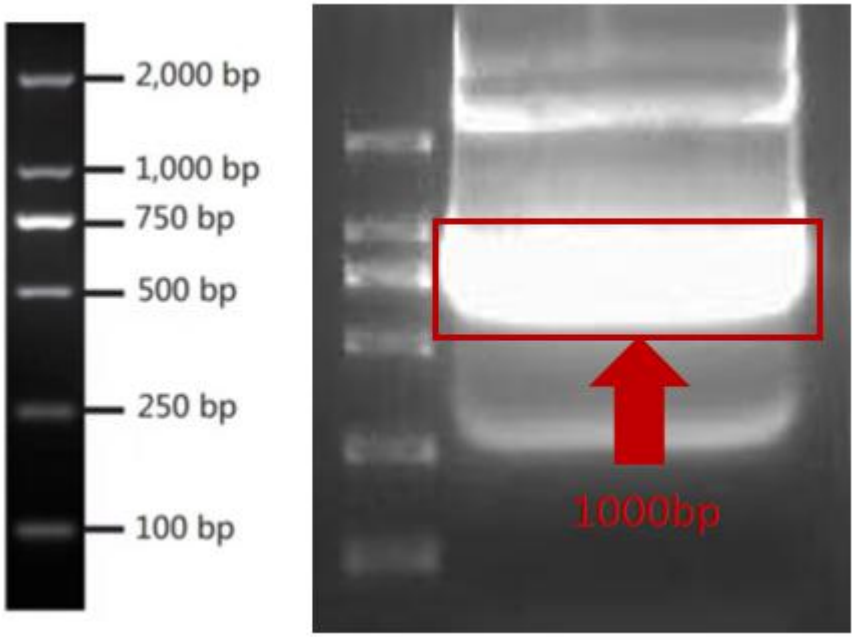
Reaction mixture for the targeting fragment（50μL）	
Donor-D500U500-trpR	1μL
ΔtrpR targeting fragment-F	2μL
ΔtrpR targeting fragment-R	2μL
2×HF Mix	25μL
ddw	220μL

Reaction program：

Reaction program for the targeting fragment		
98℃	5min	× 30
98℃	30s	
56℃	30s	
72℃	30s	
72℃	5min	
25℃	∞	

The PCR products were subjected to agarose gel electrophoresis, followed by gel extraction and purification.

The results of the agarose gel electrophoresis are as follows:



3、Preparation of the pTarget-trpR-sgRNA plasmid

Download the trpR gene sequence in GenBank (gb) format, and log in to the website https://crispy.secondarymetabolites.org/#/input to identify suitable gRNA binding sites.
Reaction mixture for plasmid amplification via PCR:

Reaction mixture for the pTarget plasmid（50μL）	
pTarget plasmid template	1μL
pTarget-trpR-F	2μL
pTarget-trpR -R	2μL
2×HF Mix	25μL
ddw	20μL

Reaction program:

Reaction program for the pTarget plasmid		
98℃	5min	× 30
98℃	30s	
46℃	30s	
72℃	1min6s	
72℃	5min	
25℃	∞	

Subsequent DpnI digestion was performed, with the digestion mixture as follows:

Digestion reaction mixture（10μL）	
pTargetz plasmid	1μL

DpnI	0.2μL
rcutsmart	1μL
ddw	7.8μL

Reaction program:

Digestion reaction program	
37℃	2h
55℃	15min
80℃	15min
25℃	∞

Using the chemical transformation method, the ligation product was transformed into DH5α competent cells, which were then spread on LB agar plates containing streptomycin (Str) resistance. The plates were incubated overnight at 37°C. Expected resistant single colonies grew on the plates, and subsequent single colonies were picked for colony PCR.
Plasmids were extracted, sent for sequencing, and the sequencing results were confirmed to be correct.

4、Electroporation

1. Take 100 μL of competent cells and add more than 500 ng of the targeting fragment and more than 300 ng of the pTarget-trpR-sgRNA plasmid. Ensure that the total volume of the plasmid and ligation product does not exceed 5 μL. Incubate on ice for 10-30 minutes.
2. Place a clean, dried 1 mm electroporation cuvette in a biosafety cabinet for 20 minutes of UV sterilization. Pre-cool the cuvette on ice, then quickly transfer the aforementioned competent cell mixture into the cuvette, ensuring the cells settle at the bottom.
3. Wipe the outer wall of the cuvette dry. Use the Ec1 electroporation program to shock the 1 mm cuvette. Immediately after electroporation, add 900-1000 μL of LB medium pre-warmed to 37°C, gently pipette to mix, and transfer the mixture to a 1.5 mL centrifuge tube. Incubate in a 37°C shaker at 150 rpm for 45-60 minutes, then spread an appropriate amount onto LB agar plates containing both kanamycin (Kana) and streptomycin (Str) antibiotics.

5、Knockout verification

PCR verification reaction mixture

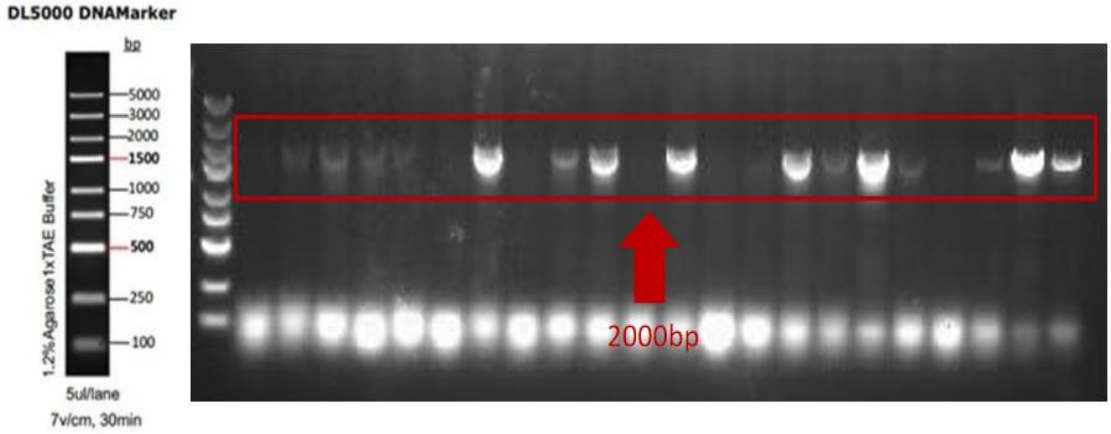
Verification reaction mixture（10μL）	
Pick a single colony	
Knockout-JP-F	0.4μL
Knockout-JP-R	0.4μL
Green Mix enzyme	5μL
ddw	4.2μL

Reaction Program:

Colony PCR Reaction Program

98℃	5min	× 30
98℃	30s	
57℃	30s	
72℃	20s	
72℃	5min	
25℃	∞	

Perform agarose gel electrophoresis to verify the knockout. The electrophoresis results are as follows:



6、Plasmid curing

Take the verified correct clones and culture them at 37°C. During the cultivation, add 10 mM rhamnose. After culturing for 2-3 hours, streak to isolate single colonies. Verify each single colony for the presence of streptomycin (Str) resistance; those without Str resistance are the clones with pTarget-trpR-sgRNA eliminated. The pEc-Cas9 plasmid can be activated by incubating at 37°C for 2-3 hours, then streaked onto agar plates containing 10 g/L sucrose.

7、Analysis and Discussion

The results showed that the trpR gene was successfully knocked out, and the strain with the knocked-out trpR gene can be prepared into chemically competent cells for subsequent experimental operations.

(C) Site-directed mutagenesis of the trpE gene

1、PCR Donor-trpE(Q71K、S94N、C495Y)

PCR System:

Donor-trpE(Q71K) Reaction System (50μL)	
Donor-trpE(Q71K)	1μL
Q71K-F	2μL
Q71K-R	2μL
2×HF Mix	25μL
ddw	20μL

Donor-trpE(S94N) Reaction System（50μL）	
Donor-trpE(S94N)	1μL
S94N -F	2μL
S94N -R	2μL
2×HF Mix	25μL
ddw	20μL

Donor-trpE(C495Y) Reaction System（50μL）	
Donor-trpE(C495Y)	1μL
C495Y -F	2μL
C495Y -R	2μL
2×HF Mix	25μL
ddw	20μL

PCR Program Setup:：

Donor-trpE(Q71K) Reaction System		
98℃	5min	
98℃	30s	× 30
56℃	30s	
72℃	130s	
72℃	5min	
25℃	∞	

Donor-trpE(S94N) Reaction Program		
98℃	5min	
98℃	30s	× 30
56℃	30s	
72℃	130s	
72℃	5min	
25℃	∞	

Donor-trpE(C495Y) Reaction Program		
98℃	5min	
98℃	30s	× 30
56℃	30s	
72℃	130s	
72℃	5min	
25℃	∞	

2、Purify the products of the Donor-trpE(Q71K, S94N, C495Y) fragments and subject them to Dpn I digestion

For product purification, transfer the sample to a 1.5 mL centrifuge tube, add 4-5 volumes of CP Buffer, and mix by pipetting. After mixing, transfer the mixture to a HiBind DNA Mini column, centrifuge at maximum speed of 15,000 g for 1 minute. Add 700 µL of DNA Wash Buffer, centrifuge at 15,000 g for 1 minute, discard the filtrate, and repeat this washing step. Then centrifuge at 15,000 g for 2 minutes, place the column in an oven to dry at 55-60°C for 5 minutes. Finally, add 30 µL of DDW or TE buffer, centrifuge at 15,000 g for 1 minute, transfer the filtrate back into the column, and centrifuge again at 15,000 g for 1 minute.
DpnI digestion reaction system:

DpnI digestion reaction system（50µL）	
Donor-trpE(Q71K、S94N、C495Y)	5µL
DpnI restriction enzyme	1µL
rCutSmart Buffer	5µL
ddw	39µL

DpnI digestion program setup:

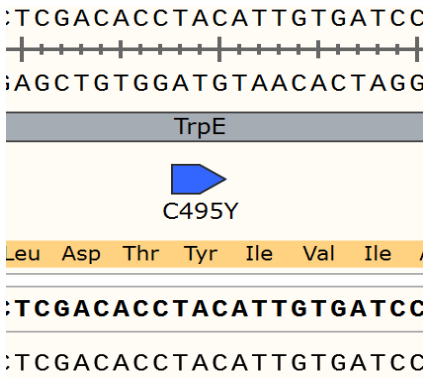
DpnI digestion program setup		
37°C	4h	
25°C		∞

3、Transform the digested products into DH5α

From the -80°C refrigerator, take 100 µL of competent cell suspension and thaw it on ice; then add all the ligation products to the suspension, shake gently, and place on ice for 20 minutes. Next, subject the mixture to heat shock in a 42°C metal bath for 45 seconds, and immediately transfer it to ice for cooling for 3-5 minutes after heat shock. After that, add 1 mL of LB liquid medium (without antibiotics) to the tube, mix well, and incubate with shaking at 37°C for 45 minutes to restore the bacteria to a normal growth state. Finally, shake the above bacterial solution evenly, take 100 µL of it to spread on a selection plate containing Str, and incubate at 37°C for 12-20 hours.

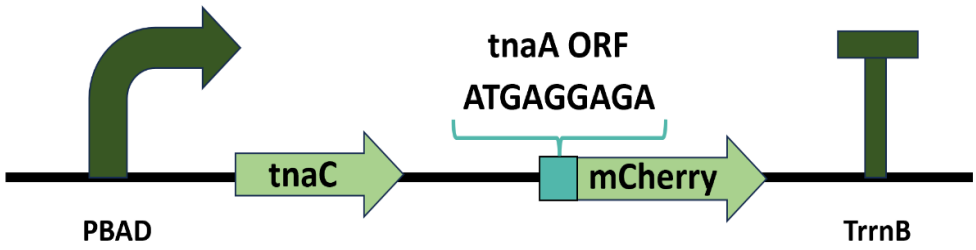
4、Extract the plasmid and send it for sequencing

The sequencing results indicated that the Q71K, S94N, and C495Y site mutations in the trpE gene were successfully achieved, and it can be ligated with the trpEDCBA gene.



(D) Test the biosensor pSB1c-PBAD-tnaC-mCherry

1、Plasmid Map



2、Transform the successfully ligated pSB1c-PBAD-tnaC-mCherry plasmid into BW25113

Take 100 μL of BW25113 competent cell suspension from the -80°C refrigerator and thaw it on ice. Add all the remaining plasmid solution to the suspension, shake gently, and place on ice for 20 minutes. Perform heat shock in a 42°C metal bath for 45 seconds, then immediately transfer to ice for cooling for 3-5 minutes. Add 1 mL of LB liquid medium (without antibiotics) to the tube, mix well, and incubate on a shaker at 37°C for 45 minutes to allow the bacteria to recover to a normal growth state. After shaking the above bacterial solution thoroughly, take 100 μL to spread on a selection plate containing chloramphenicol and incubate at 37°C for 12-20 hours.

3、Activate the bacterial strain

Pick 2/3 of the single colonies from the plate into 5 mL of LB liquid medium supplemented with 5 μL of chloramphenicol, followed by incubation on a shaker at 37°C and 200 rpm for 12 hours.

4、Inducible expression with a tryptophan gradient concentration in M9 medium

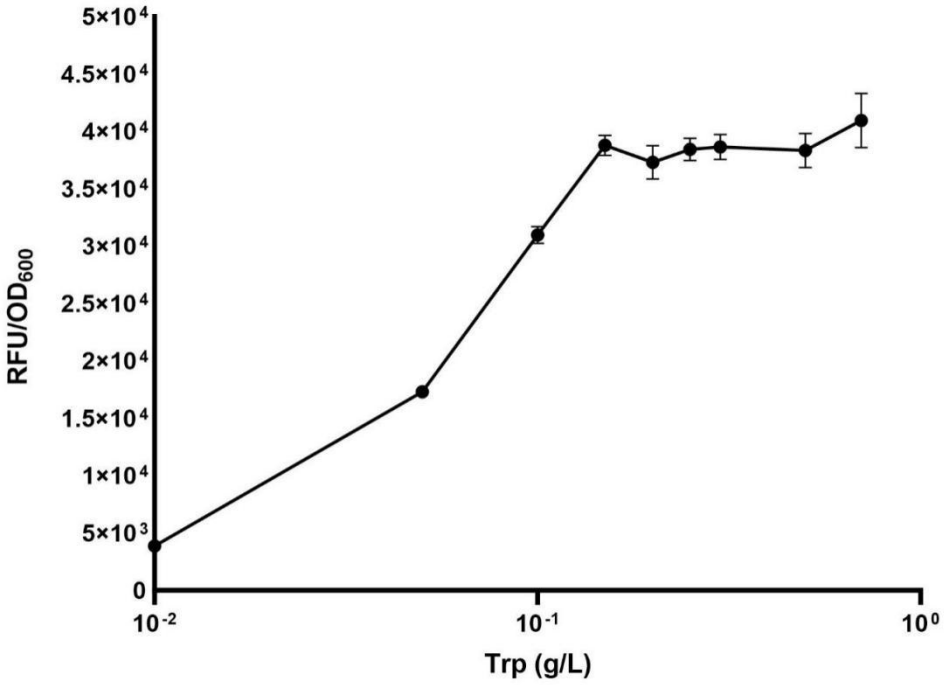
First, prepare M9 medium containing a tryptophan gradient concentration (0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.5, 0.7 g/L); then take 50 μL of the cultured BW-pSB1c-PBAD-tnaC-mCherry bacterial solution and add it to 5 mL of the prepared M9 medium; finally, incubate it on a shaker at 30°C and 200 rpm for 16 hours.

5、Microplate reader detection

Using BW25113 as the negative control, sequentially add 200 μL of the induced bacterial solution into a 96-well plate. According to the characteristics of the mCherry fluorescent protein, set a fluorescence detection program with an excitation wavelength of 552 nm and an emission wavelength of 600 nm, and simultaneously set a 600 nm wavelength to detect the OD value of the bacterial solution. Finally, calculate the ratio of fluorescence intensity (RFU) to OD600 for subsequent data analysis.

6、Data analysis

Tryptophan(g/L)	RFU/OD600 (Group 1)	RFU/OD600 (Group 2)	RFU/OD600 (Group 3)
0.01	3959.794296	3748.850046	3924.598269
0.05	17694.97000	17105.60068	17148.78175
0.10	31788.35289	30797.54601	30342.19596
0.15	39405.40541	39164.64665	37797.93185
0.20	38480.16781	37805.42071	35653.82786
0.25	39533.40799	38079.07348	37716.11526
0.30	39500.62645	37418.88337	39044.27161
0.50	39875.90324	36915.48538	38227.36642
0.70	42406.20957	42255.50831	38224.98847

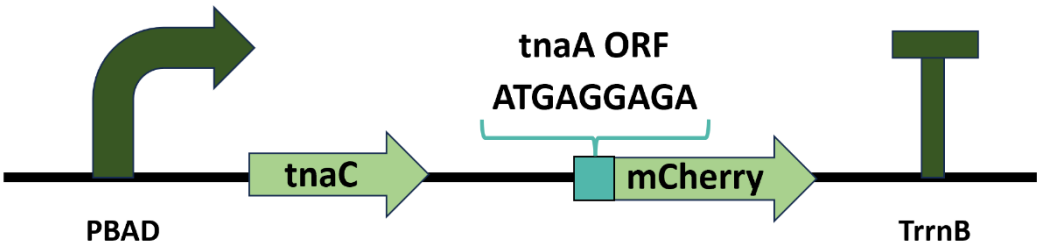


According to image analysis, the tryptophan response concentration of the pSB1c-PBAD-tnaC-mCherry biosensor is mainly concentrated in the range of 0.0-0.1 g/L tryptophan, which still needs to be improved. The next step is to continue constructing pLB1s-PBAD-tnaC-mCherry, and at the same time couple this sensor with growth to

build a sensor containing the CmR resistance gene.

(E)Construct the biosensor pLB1s-PBAD-tnaC-mCherry

1、Construct the plasmid map



2、Obtain the target gene and vector

Obtain the tnaC-mCherry fragment by PCR using pYB1a-tnaC-mCherry as a template; obtain the vector fragment by digesting pLB1s-eGFP.

PCR system:

tnaC-mCherry reaction system（50μL）	
pYB1a-tnaC-mCherry	1μL
pLB1s-F	2μL
pLB1s-R	2μL
2×HF Mix	25μL
ddw	20μL

PCR program settings:

tnaC-mCherry reaction program		
98℃	5min	
98℃	30s	× 30
58℃	30s	
72℃	33s	
72℃	5min	
25℃	∞	

pLB1s digestion system:

Digestion system （50μL）	
pLB1s-eGFP	13μL
rcutsmart	5μL
Spe I	1μL
Xho1	1μL
ddw	30μL

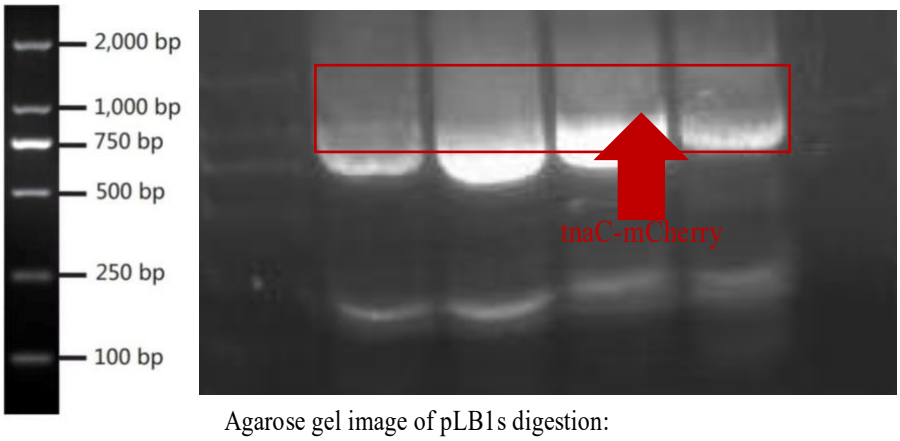
pLB1s digestion reaction program：

pLB1s digestion reaction procedure	
37℃	4h
25℃	∞

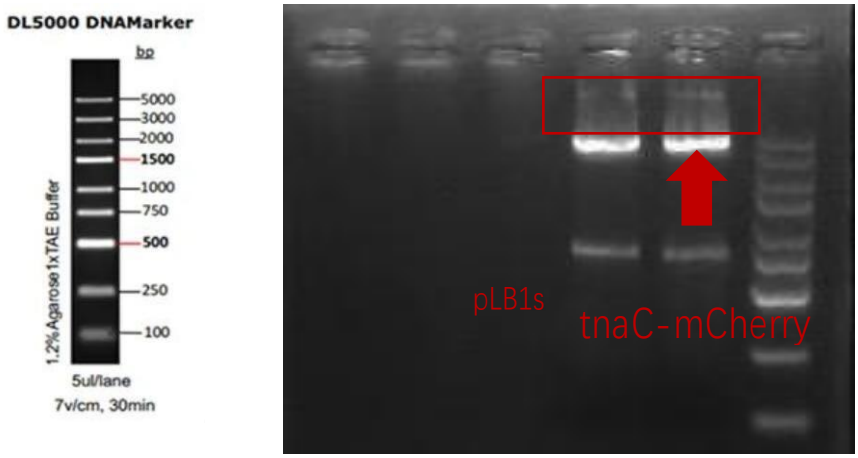
3、Gel extraction and recovery of the gene fragment

Cut and recover the correct bands obtained, then perform agarose gel electrophoresis to detect the PCR products. The results are as follows:

Electrophoretogram of tnaC-mCherry:



Agarose gel image of pLB1s digestion:



4、Gibson assembly

Ligation system:

Gibson Assembly System (10 μL)	
pLB1s vector fragment	1μL
tnaC	1μL
mCherry	1μL
2×CE Mix	5μL
ddw	2μL

Ligation procedure:

Gibson Assembly program settings	
50℃	15min
4℃	∞

5、Transform the ligation product into DH5α

Take 100 μL of competent cell suspension from the -80℃ refrigerator and thaw it on ice. Add all the ligation products to the suspension, shake gently, and place on ice for 20 minutes. Heat shock in a 42℃ metal bath for 45 seconds, then immediately transfer to ice for cooling for 3-5 minutes. Add 1 mL of LB liquid medium (without antibiotics) to the tube, mix well, and incubate with shaking at 37℃ for 45 minutes to allow the bacteria to recover to a normal growth state. After shaking the above bacterial solution thoroughly, take 100 μL and spread it on a selection plate containing ampicillin, then culture at 37℃ for 12-20 hours.

6、Colony PCR

Colony PCR system:

Colony PCR reaction system（10μL）	
Monoclonal colony	
Bacterium p-pLB1s-F	0.4μL
Bacterium p-pLB1s-R	0.4μL
Green Mix enzyme	5μL
ddw	4.2μL

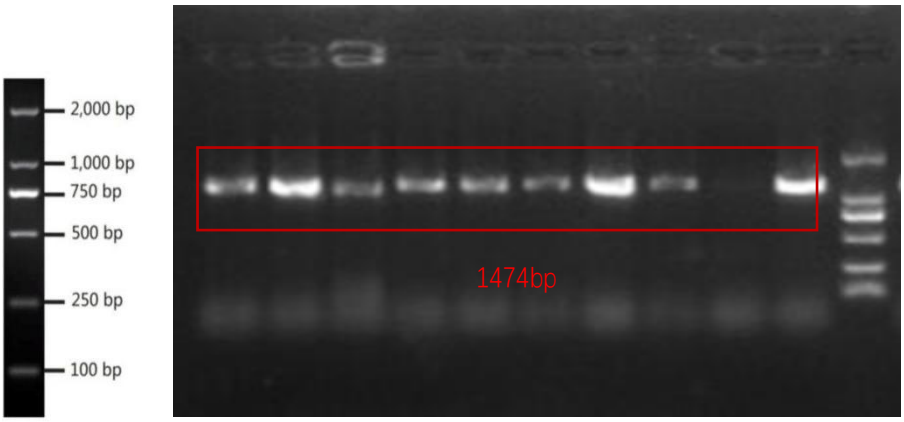
Colony PCR program settings:

Colony PCR reaction procedure			
98℃	5min		× 30
98℃	30s		
58℃	30s		
72℃	18s		
72℃	5min		
25℃		∞	

7、Agarose gel electrophoresis

Prepare a 1% agarose gel using TAE as the buffer: weigh 0.4 g of agarose powder into an Erlenmeyer flask, measure 40 ml of TAE solution to dissolve the agarose, heat it in a microwave until boiling and repeat this process two to three times to accelerate dissolution. When the temperature cools to 50-60°C, add nucleic acid dye (at a ratio of 1:10000), mix well, and pour into the gel plate to allow the gel to solidify. For sample loading, add 6× loading buffer to the samples as electrophoresis indicator. Perform electrophoresis at 120 V and stop when the loading buffer has migrated to approximately 3/4 of the gel length.

The results are as follows:



The size of the Colony PCR bands is consistent with expectations, with a positive rate of 90%.

8、Extract the recombinant plasmid

The sequencing results showed that pLB1s-PBAD-tnaC-mCherry was successfully constructed, and it can be transformed into BW25113 for induced expression and efficacy testing.

(F)CRISPR-Cas9-mediated knockout of the trpR gene

1、Preparation of electrocompetent cells

Electrocompetent cells of BW25113 harboring the pEc-Cas9 plasmid were prepared and immediately stored in a -80°C freezer.

2、Preparation of the targeting fragment

1、Fragments of 500 bp each from the upstream and downstream regions of the trpR gene, designated as D500 and U500 respectively, were obtained by PCR. Meanwhile, the Donor vector was also amplified via PCR. The PCR system and procedure are as follows:

The U500 reaction system（50μL）	
Bacterial culture (broth)	1μL
trpR-U500-F	2μL
trpR-U500-R	2μL
2×HF Mix	25μL
ddw	20μL

The D500 reaction system（50μL）	
Bacterial culture (broth)	1μL
trpR-D500-F	2μL
trpR-D500-R	2μL
2×HF Mix	25μL
ddw	20μL

The Donor reaction system（50μL）	
The Donor plasmid	1μL
trpR-Do-F	2μL
trpR-Do- R	2μL
2×HF Mix	25μL
ddw	20μL

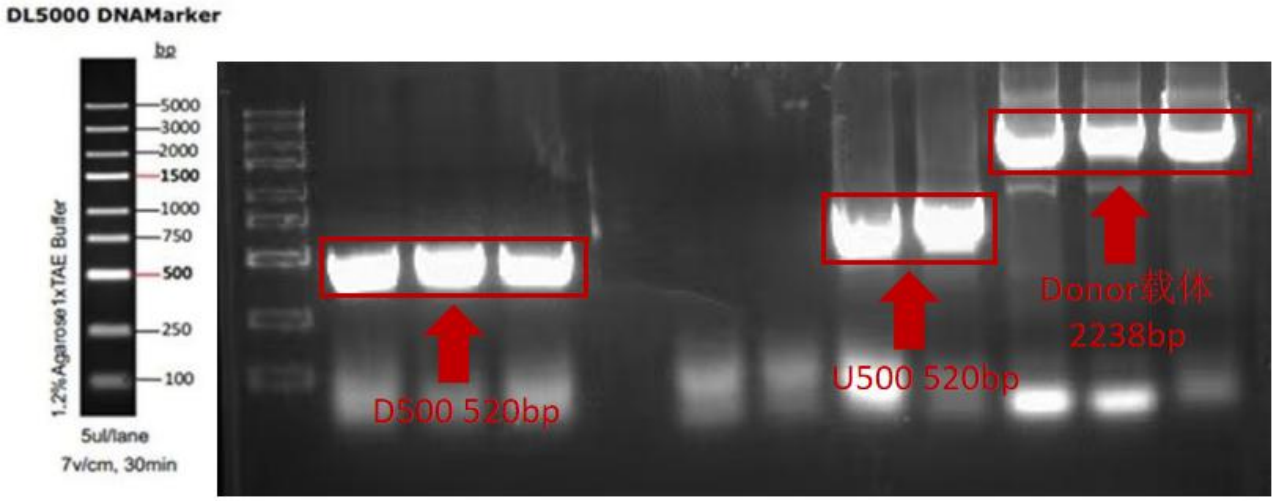
The U500 reaction program		
98℃	5min	
98℃	30s	× 30
56℃	30s	
72℃	30s	
72℃	5min	
25℃	∞	

The D500 reaction program		
98℃	5min	× 30
98℃	30s	
56℃	30s	
72℃	30s	
72℃	5min	
25℃	∞	

The Donor reaction program		
98℃	5min	× 30
98℃	30s	
55℃	30s	
72℃	1min9s	
72℃	5min	
25℃	∞	

Subsequently, the PCR products were subjected to agarose gel electrophoresis, followed by gel extraction and purification.

The results of agarose gel electrophoresis are as follows:



Then, we performed Gibson assembly on several fragments.

The assembly system is as follows:

Gibson assembly system（10μL）	
The Donor vector	1.4μL
U500	1.2μL
D500	1.4μL
2×CE Mix	5μL
ddw	1μL

The Gibson assembly program is as follows：

Gibson assembly program settings	
55℃	30min
4℃	∞

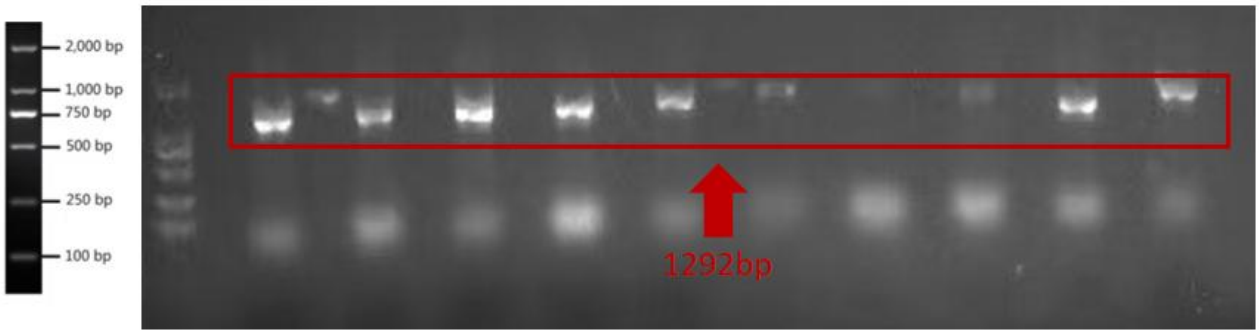
We performed chemical transformation by introducing the ligation products into DH5α competent cells, followed by plating onto LB agar plates supplemented with streptomycin (Str) selection marker. The plates were then incubated overnight at 37°C in a humidified incubator. Expected streptomycin-resistant single colonies emerged on the plates. Subsequently, individual colonies were picked for colony PCR screening to verify positive clones. The colony PCR reaction mixture is as follows:

The reaction mixture for colony PCR （10μL）	
Picking single colonies	
DO-trpR-JP-F	0.4μL
DO-trpR-JP -R	0.4μL
Green Mix enzyme	5μL
ddw	4.2μL

The reaction program for colony PCR is as follows:

The reaction program for colony PCR		
98℃	5min	
98℃	30s	× 30
57℃	30s	
72℃	20s	
72℃	5min	
25℃	∞	

The colony PCR results were analyzed by agarose gel electrophoresis. The results are as follows:



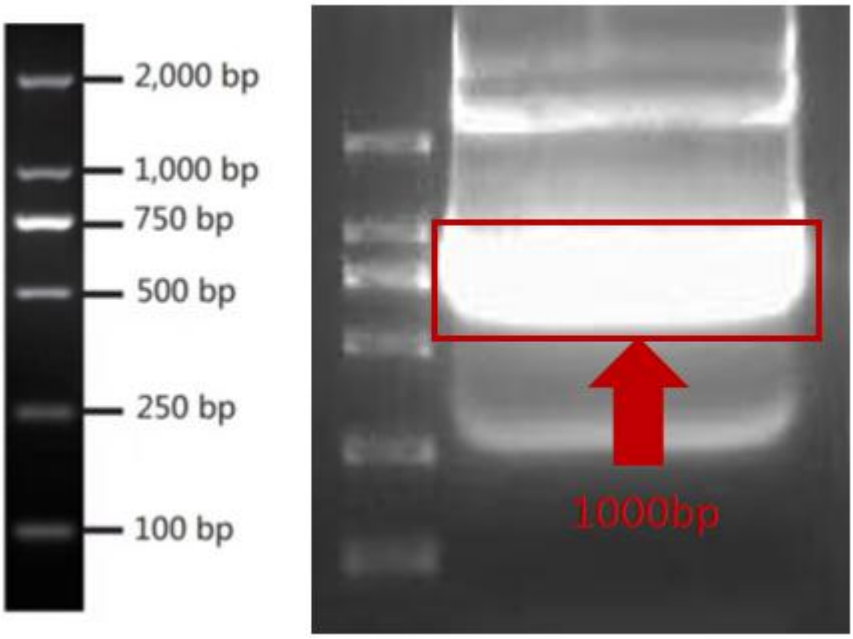
Subsequently, the samples were sent for sequencing, and the results were confirmed to be correct.
Reaction mixture for obtaining linear D500 and U500 fragment via PCR:

Reaction mixture for the targeting fragment（50μL）	
Donor-D500U500-trpR	1μL
ΔtrpR targeting fragment-F	2μL
ΔtrpR targeting fragment-R	2μL
2×HF Mix	25μL
ddw	220μL

Reaction program：

Reaction program for the targeting fragment		
98℃	5min	
98℃	30s	× 30
56℃	30s	
72℃	30s	
72℃	5min	
25℃	∞	

The PCR products were subjected to agarose gel electrophoresis, followed by gel extraction and purification. The results of the agarose gel electrophoresis are as follows：



3、Preparation of the pTarget-trpR-sgRNA plasmid

Download the trpR gene sequence in GenBank (gb) format, and log in to the website https://crispy.secondarymetabolites.org/#/input to identify suitable gRNA binding sites.

Reaction mixture for plasmid amplification via PCR:

Reaction mixture for the pTarget plasmid（50μL）	
pTarget plasmid template	1μL
pTarget-trpR-F	2μL
pTarget-trpR -R	2μL
2×HF Mix	25μL
ddw	20μL

Reaction program：

Reaction program for the pTarget plasmid		
98℃	5min	× 30
98℃	30s	
46℃	30s	
72℃	1min6s	
72℃	5min	
25℃	∞	

Subsequent DpnI digestion was performed, with the digestion mixture as follows:

Digestion reaction mixture（10μL）	
pTargetz plasmid	1μL
DpnI	0.2μL
rcutsmart	1μL
ddw	7.8μL

Reaction program：

Digestion reaction program	
37℃	2h
55℃	15min
80℃	15min
25℃	∞

Using the chemical transformation method, the ligation product was transformed into DH5α competent cells, which were then spread on LB agar plates containing streptomycin (Str) resistance. The plates were incubated overnight at 37°C. Expected resistant single colonies grew on the plates, and subsequent single colonies were picked for colony PCR.

Plasmids were extracted, sent for sequencing, and the sequencing results were confirmed to be correct.

4、Electroporation

1. Take 100 μL of competent cells and add more than 500 ng of the targeting fragment and more than 300 ng of

the pTarget-trpR-sgRNA plasmid. Ensure that the total volume of the plasmid and ligation product does not exceed 5 μL. Incubate on ice for 10-30 minutes.

2. Place a clean, dried 1 mm electroporation cuvette in a biosafety cabinet for 20 minutes of UV sterilization. Pre-cool the cuvette on ice, then quickly transfer the aforementioned competent cell mixture into the cuvette, ensuring the cells settle at the bottom.

3. Wipe the outer wall of the cuvette dry. Use the Ec1 electroporation program to shock the 1 mm cuvette. Immediately after electroporation, add 900-1000 μL of LB medium pre-warmed to 37°C, gently pipette to mix, and transfer the mixture to a 1.5 mL centrifuge tube. Incubate in a 37°C shaker at 150 rpm for 45-60 minutes, then spread an appropriate amount onto LB agar plates containing both kanamycin (Kana) and streptomycin (Str) antibiotics.

5、Knockout verification

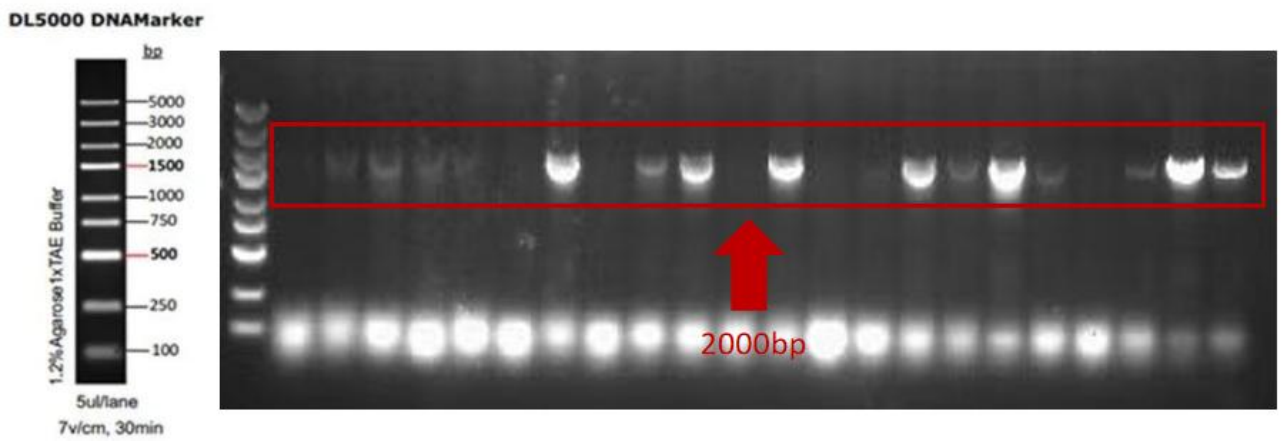
PCR verification reaction mixture

Verification reaction mixture（10μL）	
Pick a single colony	
Knockout-JP-F	0.4μL
Knockout-JP-R	0.4μL
Green Mix enzyme	5μL
ddw	4.2μL

Reaction Program：

Colony PCR Reaction Program		
98℃	5min	
98℃	30s	× 30
57℃	30s	
72℃	20s	
72℃	5min	
25℃	∞	

Perform agarose gel electrophoresis to verify the knockout. The electrophoresis results are as follows:



6、Plasmid curing

Take the verified correct clones and culture them at 37°C. During the cultivation, add 10 mM rhamnose. After culturing for 2-3 hours, streak to isolate single colonies. Verify each single colony for the presence of streptomycin (Str) resistance; those without Str resistance are the clones with pTarget-trpR-sgRNA eliminated. The pEc-Cas9 plasmid can be activated by incubating at 37°C for 2-3 hours, then streaked onto agar plates containing 10 g/L sucrose.

7、Analysis and Discussion

The results showed that the trpR gene was successfully knocked out, and the strain with the knocked-out trpR gene can be prepared into chemically competent cells for subsequent experimental operations.

(G) Point mutation in the trpE gene

1PCR Donor-trpE(Q71K、S94N、C495Y)

PCR system：

Donor-trpE(Q71K) Reaction system（50μL）	
Donor-trpE(Q71K)	1μL
Q71K-F	2μL
Q71K-R	2μL
2×HF Mix	25μL
ddw	20μL

Donor-trpE(S94N) Reaction system（50μL）	
Donor-trpE(S94N)	1μL
S94N -F	2μL
S94N -R	2μL
2×HF Mix	25μL
ddw	20μL

Donor-trpE(C495Y) Reaction system（50μL）	
Donor-trpE(C495Y)	1μL
C495Y -F	2μL
C495Y -R	2μL
2×HF Mix	25μL
ddw	20μL

PCRProgram Settings:

Donor-trpE(Q71K) Reaction Procedure		
98℃	5min	
98℃	30s	× 30
56℃	30s	
72℃	130s	
72℃	5min	
25℃	∞	

Donor-trpE(S94N) Reaction Procedure		
98℃	5min	
98℃	30s	× 30
56℃	30s	
72℃	130s	
72℃	5min	
25℃	∞	

Donor-trpE(C495Y) Reaction Procedure		
98℃	5min	
98℃	30s	× 30
56℃	30s	
72℃	130s	
72℃	5min	
25℃	∞	

Week4：3.22-3.28

(A) Knockout of tnaA and tnaB genes using CRISPR-Cas9

I.Preparation of Electrocompetent Cells

1. Inoculate bacterial solution from the glycerol stock of BW25113-ΔtrpR containing pEc-Cas9 plasmid stored in the laboratory onto a Kana-resistant LB agar plate for streaking isolation. Incubate at 37°C for 12 hours. Pick a single colony and transfer it to 5 mL of LB liquid medium. Culture at 37°C with shaking at 200 rpm for 12 hours, then inoculate 1% of the culture into 100 mL of LB medium. After 0.5 hours of incubation, add arabinose to a final concentration of 0.2%.

2. Incubate at 30°C for approximately 2–2.5 hours until the OD_{600} reaches 0.55–0.6. Remove the culture and place it on ice for 30 minutes to chill.

3. Pre-cool a centrifuge and centrifuge tubes at 4°C. Transfer 25 mL of the culture to pre-chilled centrifuge tubes, seal with parafilm, and centrifuge at 4,200 rpm for 10 minutes at 4°C. Discard the supernatant.

4. Resuspend the cell pellet in 20 mL of ice-cold sterile 10% glycerol (gently rub the tube on ice to ensure uniform resuspension). Keep the suspension on ice, then centrifuge at 4,200 rpm for 10 minutes at 4°C.

5. Immediately discard the supernatant after centrifugation. Gently resuspend the pellet and maintain it on ice throughout the process.

6. Repeat steps 4 and 5 for three times. Use 20 mL of ice-cold sterile 10% glycerol for the first two washes, and

resuspend the cells in 0.5 mL of sterile 10% glycerol for the final wash.

7. Aliquot the cell suspension into pre-chilled centrifuge tubes at 100 μL per tube. Use immediately for electroporation or store at -80°C.

II.Preparation of Targeting Fragments

1、PCR

Amplify two 500 bp fragments flanking the *tnaAB* gene (designated as D500 and U500, representing the downstream and upstream homologous arms, respectively) via PCR. Additionally, amplify the Donor vector using PCR. The PCR reaction system and cycling program are as follows:

U500 PCR Reaction System（50μL）	
Bacterial	1μL
tna-U500-F	2μL
tna-U500-R	2μL
2×HF Mix	25μL
ddw	20μL

D500 PCR Reaction System（50μL）	
Bacterial	1μL
tna-D500-F	2μL
tna-D500-R	2μL
2×HF Mix	25μL
ddw	20μL

Donor PCR Reaction System（50μL）	
Donor Plasmid	1μL
tna-Do-F	2μL
tna-Do-R	2μL
2×HF Mix	25μL
ddw	20μL

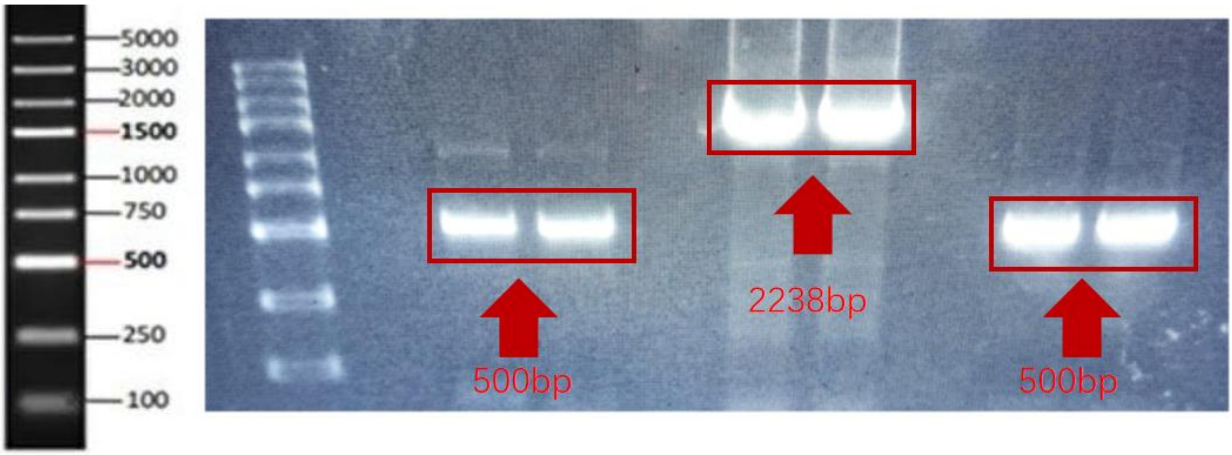
U500 PCR Cycling Program		
98℃	5min	
98℃	30s	⎫
56℃	30s	⎬ × 30
72℃	30s	⎭
72℃	5min	
25℃	∞	

D500 PCR Cycling Program		
98℃	5min	
98℃	30s	× 30
54℃	30s	
72℃	30s	
72℃	5min	
25℃	∞	

Donor PCR Cycling Program		
98℃	5min	
98℃	30s	× 30
55℃	30s	
72℃	1min9s	
72℃	5min	
25℃	∞	

3. Gel Extraction and Purification

Excise and recover the correct bands obtained. The PCR products were analyzed by agarose gel electrophoresis, and the results are as follows:



4. Gibson Assembly

Gibson Assembly：

Gibson Assembly（10μL）	
Donor plasmid	1.4μL
U500	1.2μL
D500	1.4μL
2×CE Mix	5μL
ddw	1μL

Gibson Assembly Program：

Gibson Assembly Program	
55℃	30min
4℃	∞

5. Transformation of Ligation Products into DH5αCompetent Cells

(1) Retrieve 100 μL of competent cell suspension from the -80°C freezer and allow it to thaw on ice.

(2) Add the entire volume of the ligation product to the cell suspension, gently mix, and incubate on ice for 20 minutes.

(3) Heat-shock the mixture in a 42°C metal bath for 45 seconds, then immediately transfer to ice for cooling for 3–5 minutes.

(4) Add 1 mL of antibiotic-free LB liquid medium to the tube, mix well, and incubate at 37°C with shaking for 45 minutes to allow bacterial recovery and normal growth.

(5) After mixing the bacterial culture thoroughly, take 100 μL and spread it onto a selection plate containing ampicillin. Incubate at 37°C for 12–20 hours.

6、Colony PCR

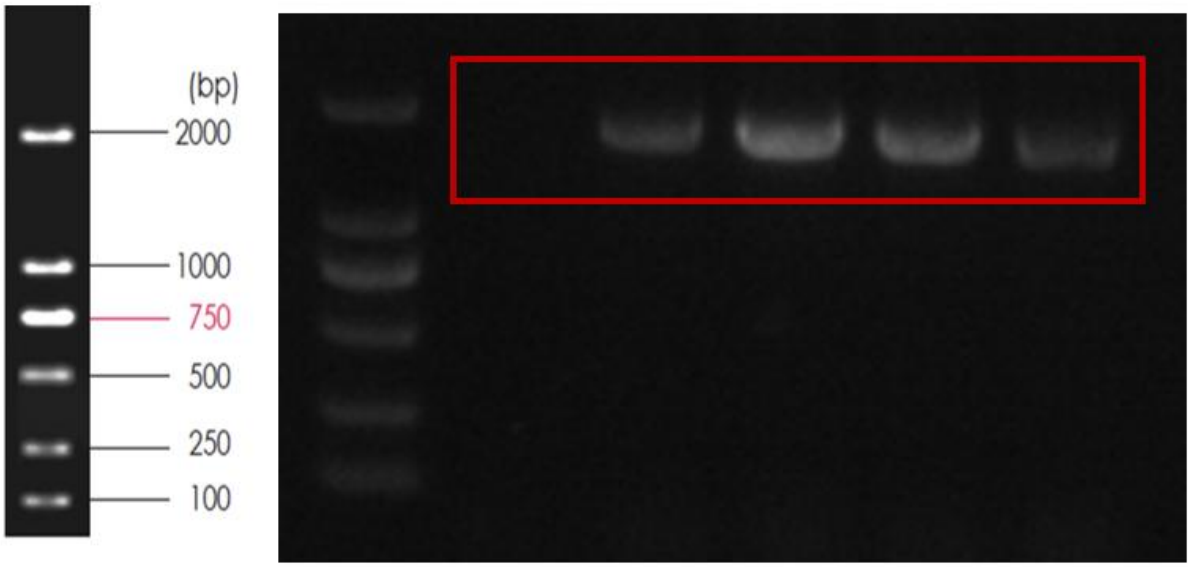
Colony PCR Reaction:

Colony PCR Reaction （10μL）	
Pick a single colony	
DO-tna-JP-F	0.4μL
DO-tna-JP -R	0.4μL
Green Mix	5μL
ddw	4.2μL

Colony PCR Colony PCR program：

Colony PCR Colony PCR program		
98℃	5min	
98℃	30s	× 30
60℃	30s	
72℃	20s	
72℃	5min	
25℃	∞	

Agarose gel electrophoresis was performed to verify the colony PCR results, and the results are as follow:



7、sequencing The sequencing results were confirmed to be correct.

8、Amplify the linear D500U500 fragment by PCR：

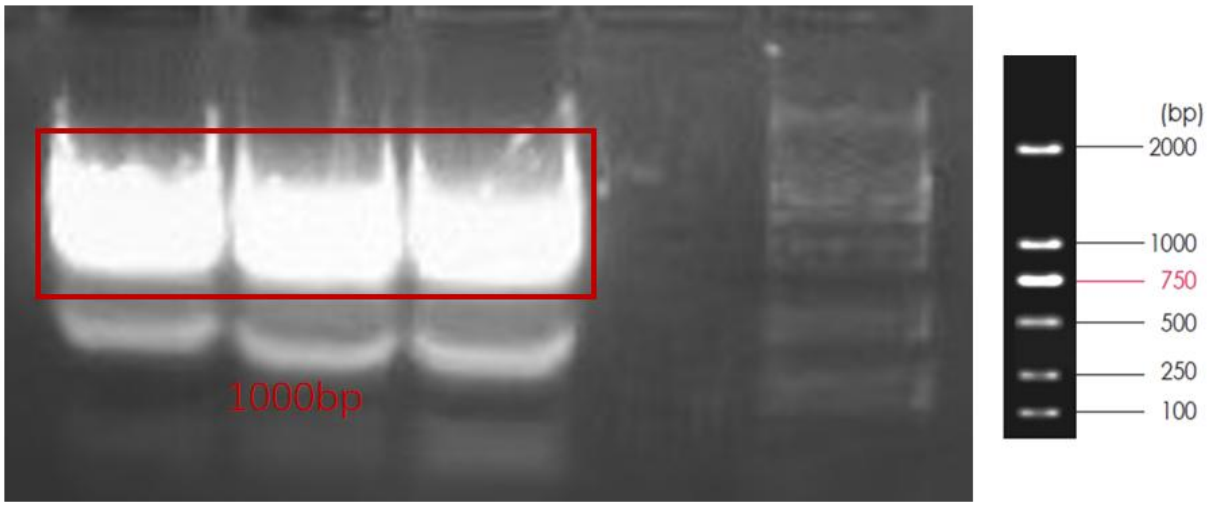
Targeting fragment reaction system（50μL）	
Donor-D500U500-tna	1μL
Δtna 打靶片段-F	2μL
Δtna 打靶片段-R	2μL
2×HF Mix	25μL
ddw	220μL

reaction program：

Targeting fragment reaction program		
98℃	5min	
98℃	30s	× 30
58℃	30s	
72℃	30s	
72℃	5min	
25℃	∞	

9、Gel Extraction and Purification

Excise and recover the correct bands obtained. The PCR products were subjected to agarose gel electrophoresis, and the results are as follows:



III.Preparation of pTarget-trpR-sgRNA Plasmid

1、Download the gene sequences of tnaA and tnaB in GenBank (gb) format.

Access the online tool via https://crispy.secondarymetabolites.org/#/input to identify suitable gRNA target sites with high affinity.

2、Amplify the plasmid by PCR

Reaction system:

pTarget Reaction system（50μL）	
pTarget	1μL
pTarget-tna-F	2μL
pTarget-tna-R	2μL
2×HF Mix	25μL
ddw	20μL

Reaction program:

pTarget Reaction program		
98℃	5min	
98℃	30s	× 30
46℃	30s	
72℃	1min6s	
72℃	5min	
25℃	∞	

3.DpnI Digestion

Digestion System：

Digestion Reaction System（10μL）	
pTarget	1μL

DpnI	0.2μL
rcutsmart	1μL
ddw	7.8μL

Reaction program:

Digestion Reaction program:		
37℃	2h	× 30
55℃	15min	
80℃	15min	
25℃	∞	

4、Transformation of Ligation Products into DH5α

Using the chemical transformation method, transfer the ligation products into DH5α competent cells, then spread them onto an LB plate containing Str (streptomycin) resistance, and incubate at 37°C overnight. Antibiotic-resistant single colonies as expected grow on the plate, and single colonies will be selected for colony PCR in the subsequent step.

5、Plasmid extraction and sequencing submission

The sequencing results showed that the tnaA and tnaB genes were successfully knocked out, and this strain can be prepared into chemically competent cells for experimental operations.

IV. Electroporation

1. Take 100 μL of competent cells and add more than 500 ng of the targeting fragment and more than 300 ng of the pTarget-tna-sgRNA plasmid. Ensure the total volume of the plasmid and ligation product does not exceed 5 μL. Incubate on ice for 10–30 minutes.
2. Place a cleaned, dried 1 mm electroporation cuvette in a laminar flow hood for 20 minutes of UV sterilization. Pre-cool the cuvette on ice, then quickly transfer the above competent cell mixture into the cuvette, ensuring the cells settle at the bottom of the cuvette.
3. Wipe the outer wall of the cuvette dry. Use the Ec1 electroporation program for the 1 mm cuvette. Immediately after electroporation, add 900–1000 μL of LB medium pre-warmed to 37°C, gently pipette to mix, and transfer to a 1.5 mL centrifuge tube. Incubate in a 37°C shaker at 150 rpm for 45–60 minutes, then spread an appropriate amount onto LB plates containing both kanamycin (Kana) and streptomycin (Str) for double-antibiotic selection.

V. Knockout Verification

1.PCR Verification

Reaction System:

Verification Reaction System（10μL）	
Pick a single colony	
Knockout -JP-F	0.4μL
Knockout -JP -R	0.4μL

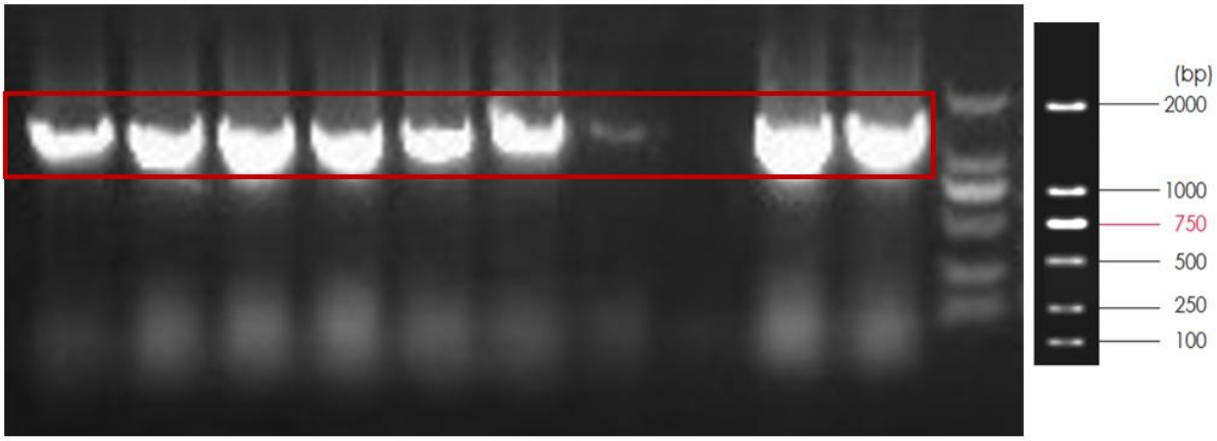
Green Mix	5μL
ddw	4.2μL

Reaction program：

Colony PCR Reaction program		
98℃	5min	
98℃	30s	× 30
57℃	30s	
72℃	20s	
72℃	5min	
25℃	∞	

Agarose gel electrophoresis verification.

The results are as follows:

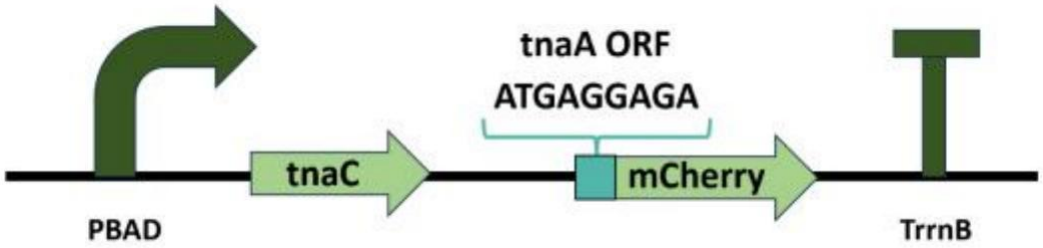


VI. Plasmid Elimination

Take the correctly verified clones and culture them at 37°C. During cultivation, add 10 mM rhamnose and incubate for 2–3 hours, then streak to isolate single colonies. Verify each single colony for the presence of Str resistance; colonies without Str resistance are those with the pTarget-tna-sgRNA plasmid eliminated. The pEc-Cas9 plasmid can be eliminated by activating (culturing) at 37°C for 2–3 hours, then streaking onto agar plates containing 10 g/L sucrose.

(A) Testing the Biosensor pLB1s-PBAD-tnaC-mCherry

I. Plasmid Map



II. Transformation of Successfully Ligated pLB1s-PBAD-tnaC-mCherry Plasmid into BW25113

1. Take 100 μL of BW25113 competent cell suspension from the -80°C refrigerator and thaw it on ice.
2. Add the entire remaining plasmid solution to the suspension, gently shake to mix, and incubate on ice for 20 minutes.
3. Heat-shock the mixture in a 42°C metal bath for 45 seconds, then immediately transfer to ice for cooling for 3–5 minutes.
4. Add 1 mL of LB liquid medium (without antibiotics) to the tube, mix well, and incubate in a 37°C shaker for 45 minutes to allow the bacteria to recover to their normal growth state.
5. After shaking the bacterial solution thoroughly, take 100 μL and spread it onto a selection plate containing streptomycin. Incubate at 37°C for 12–20 hours.

III. Strain Activation

1. Pick 2/3 of a single colony from the plate into 5 mL of LB liquid medium, and add 5 μL of streptomycin.
2. Incubate in a shaker at 37°C, 200 rpm for 12 hours.

IV. Induced Expression in M9 Medium with Gradient Concentrations of Tryptophan

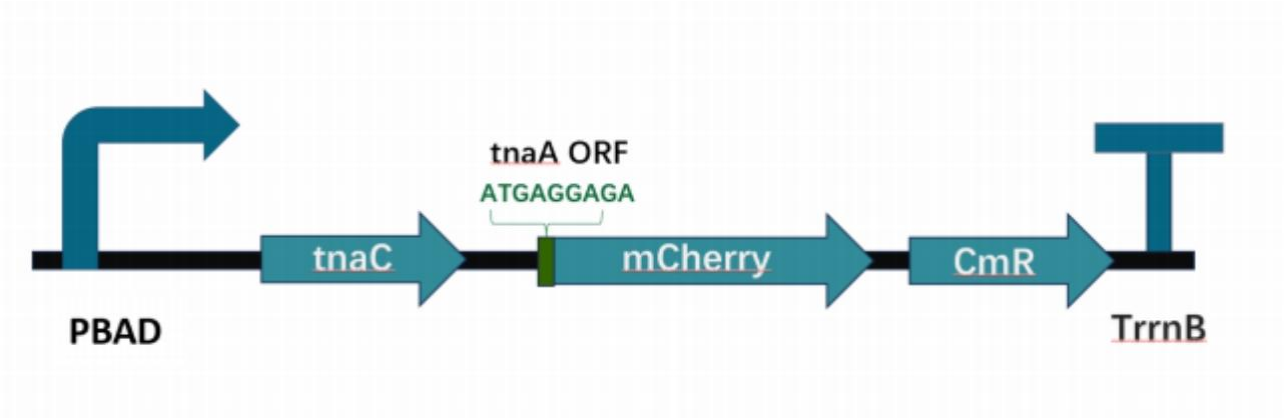
1. Prepare M9 medium containing gradient concentrations of tryptophan (0.01, 0.2, 0.4, 0.6, 0.8, 1.2, 1.4, 1.6 g/L).
2. Take 50 μL of the cultured BW-pLB1s-PBAD-tnaC-mCherry bacterial solution and add it to 5 mL of the prepared M9 medium.
3. Incubate in a shaker at 30°C, 200 rpm for 16 hours.

V. Microplate Reader Detection

1. Add 200 μL of the induced bacterial solution to a 96-well plate in sequence.
2. According to the characteristics of the mCherry fluorescent protein, set a fluorescence detection program with an excitation wavelength of 552 nm and an emission wavelength of 600 nm. Additionally, set a wavelength of 600 nm to detect the OD value of the bacterial solution.
3. Finally, calculate the ratio of fluorescence intensity (RFU) to OD600 for data analysis.

(B) Construction of the Biosensor pLB1s-PBAD-tnaC-mCherry-CmR

I.Construction of the Plasmid Map



II. Acquisition of Target Genes and Vector

Amplify the tnaC-mCherry fragment by PCR using pYB1a-tnaC-mCherry as the template; amplify the CmR (chloramphenicol resistance) fragment by PCR using pSB1c-eGFP as the template; obtain the vector fragment by digesting pLB1s-eGFP.

PCR system:

tnaC-mCherry PCR system（50μL）	
PYB1a-tnaC-mCherrry	1μL
tnaC-F	2μL
mCherrry -R	2μL
2×HF Mix	25μL
ddw	20μL

CmR PCR system（50μL）	
pSB1c-eGFP	1μL
CmR-F	2μL
CmR-R	2μL
2×HF Mix	25μL
ddw	20μL

PCR Program：

tnaC-mCherry system		
98℃	5min	
98℃	30s	× 30
55℃	30s	
72℃	33s	
72℃	5min	
25℃	∞	

CmR PCR Program		
98℃	5min	
98℃	30s	× 30
55℃	30s	
72℃	30s	
72℃	5min	
25℃	∞	

Enzyme Digestion System for pLB1s:

Enzyme Digestion System（50μL）	
pLB1s-eGFP	13μL
rcutsmart	5μL
SpeI	1μL
Xho1	1μL
ddw	30μL

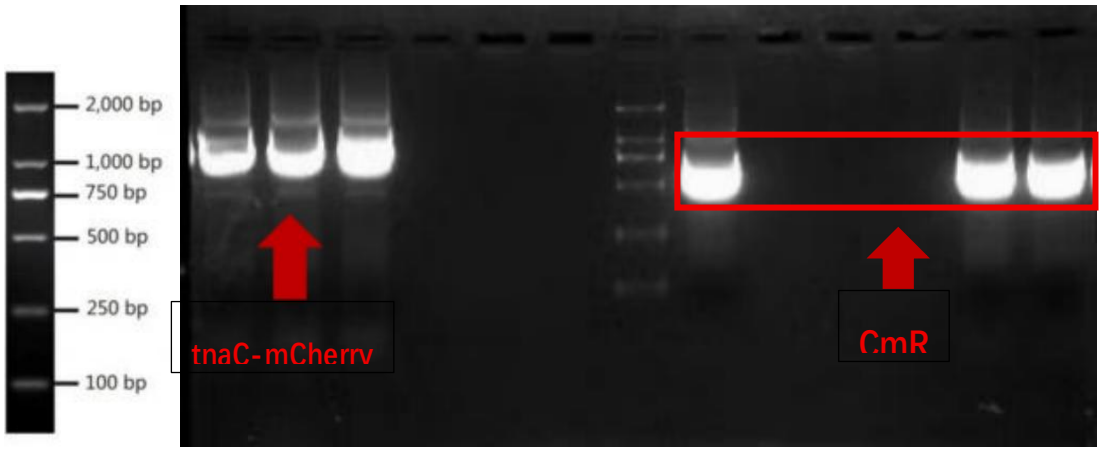
pLB1s Enzyme Digestion Program：

pLB1s Enzyme Digestion Program	
37℃	4h
25℃	∞

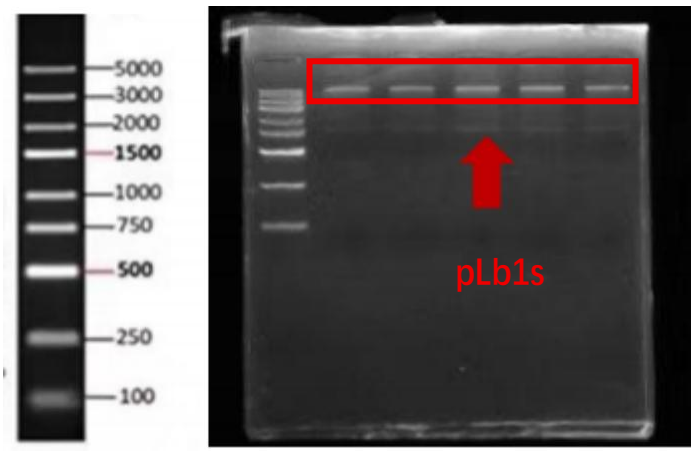
III. Gel Extraction of Gene Fragments

Agarose gel electrophoresis was performed on the PCR products, followed by gel extraction.

Electrophoretogram of tnaC-mCherry and CmR fragments:



pLB1s Enzyme Digestion Electrophoresis Pattern：



IV. Gibson Assembly

Ligation System:

Ginson Ligation System（10μL）	
pLB1s	5.2μL
tnaC-mChrry	1.6μL
CmR	1.5μL
2×CE Mix	5μL
ddw	2μL

Due to the low concentration of the enzyme-digested vector, the ligation system was slightly scaled up.

Ligation procedure:

Gibson Ligation procedure:	
50℃	30min
4℃	∞

V. Transformation of Ligation Products into DH5α

Using the chemical transformation method, transfer the ligation products into DH5α competent cells, then spread them onto an LB plate containing streptomycin (Str) resistance, and incubate at 37°C overnight. The expected antibiotic-resistant single colonies grow on the plate, and single colonies will be selected for colony PCR in subsequent steps.

VI. Colony PCR

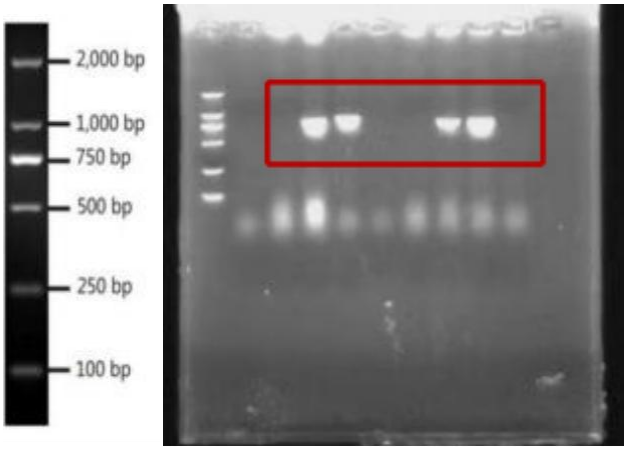
Colony PCR system:

Colony PCR system（10μL）	
Single colony	
JP-pLB1s-F	0.4μL
JP-pLB1s-R	0.4μL
Green Mix	5μL
ddw	2μL

Colony PCR Program:

Colony PCR Program		
98℃	5min	
98℃	30s	× 30
58℃	30s	
72℃	15s	
72℃	5min	
25℃	∞	

Agarose gel electrophoresis was performed on the colony PCR results.
The results are as follows:



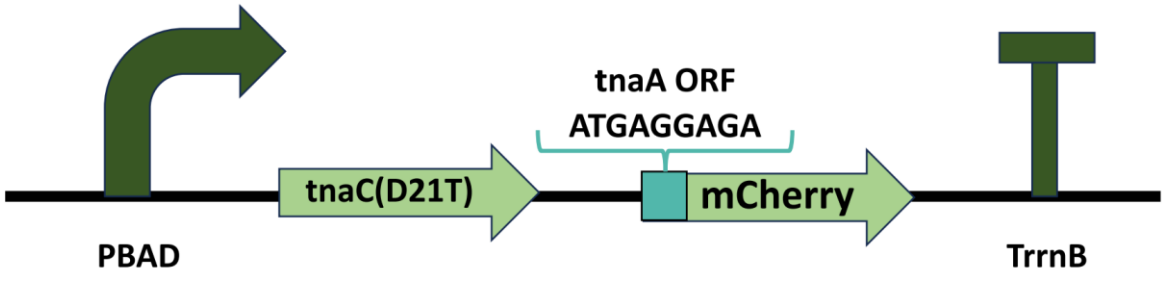
The size of the colony PCR bands is consistent with expectations, and the positive rate is 40%.

VII. Extraction of recombinant plasmids

Sequencing results showed that pLB1s-PBAD-tnaC-mCherry-CmR was successfully constructed and can be transformed into BW25113 for induced expression and effect testing.

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

I. Plasmid Map



II. 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

A. Detecting the impact of trpR and tnaAB gene knockouts on tryptophan production

I. 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 MgCl2, 20mM CaCl2) 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 CaCl2), 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.

II. 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.

III. 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.

IV. 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.

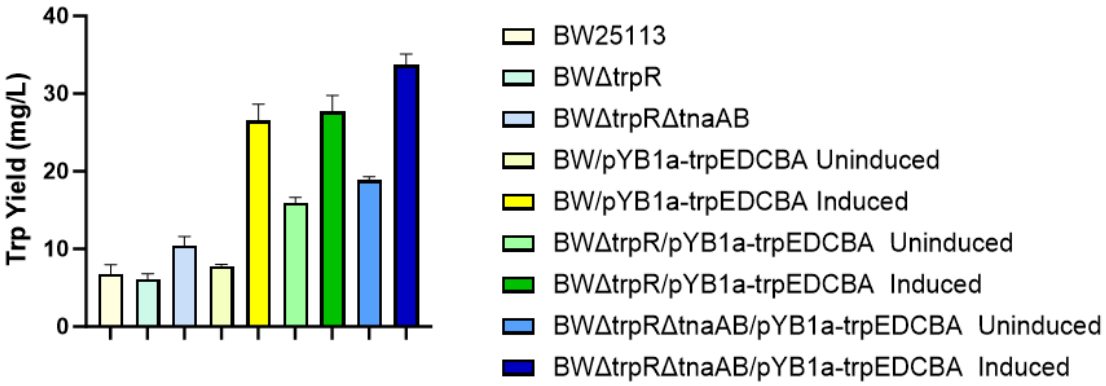
③ Inject different samples into the HPLC system in sequence, with a flow rate of 1.0mL/min and an injection volume of 10μL.

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

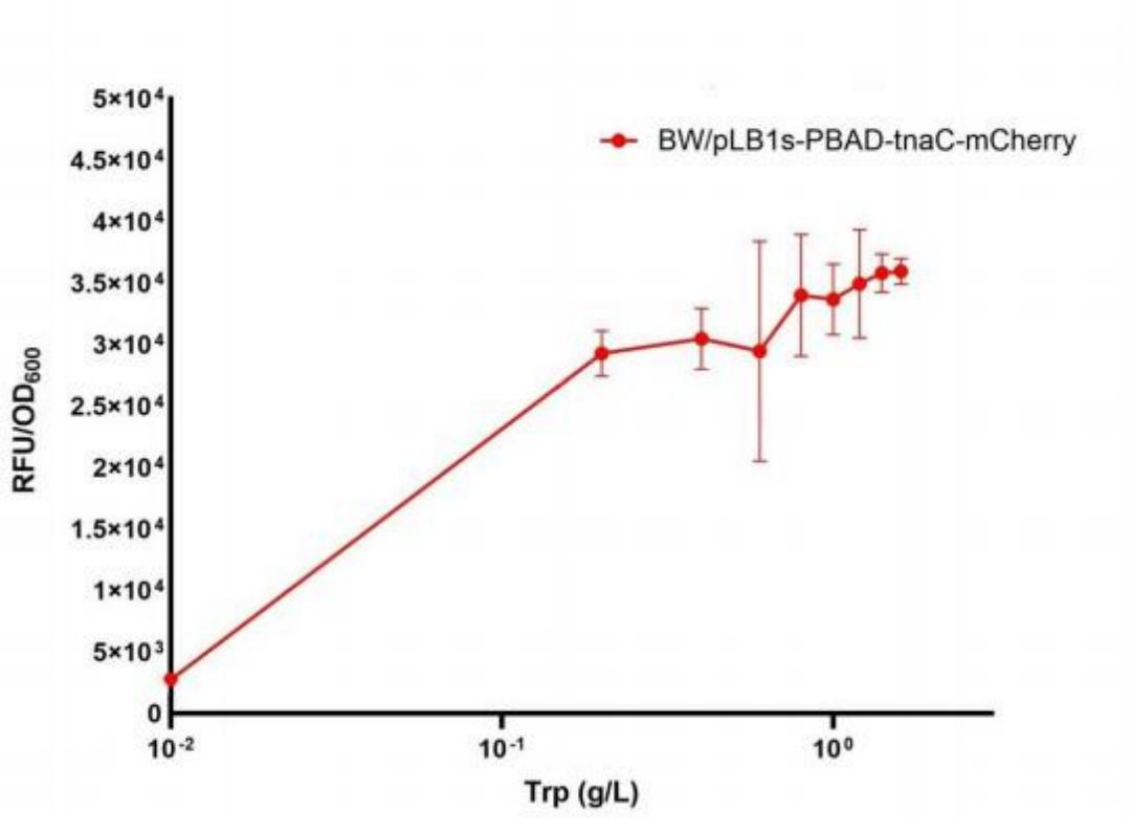


V. Analysis and Discussion

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-ΔtrpR.

VI. 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.