The experimental objective in June: Construct four new plasmids, test sensor response to tryptophan, and evaluate tryptophan-to-melatonin pathways via plasmid assembly.

Week1: 6.1-6.7:

(A)Construction of pSB1c-SgAANAT-CrTDC plasmid

1. Amplification of vectors by PCR

PCR system (50μL)	
2×Mix	25μl
pLB1s-F	2μl
pLB1s-R	2μl
template	50ng
DDW	to 50μl

PCR		
98℃	5min	
98℃	30s	×30
62℃	30s	
72℃	60s	
72℃	5min	
25℃	∞	

2.Amplification of SgAANAT fragments by PCR

PCR system (50μL)	
2×Mix	25μl
SgAANAT-F	2μl
×30 AANAT-R	2μl
template	50ng
DDW	to 50μl

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

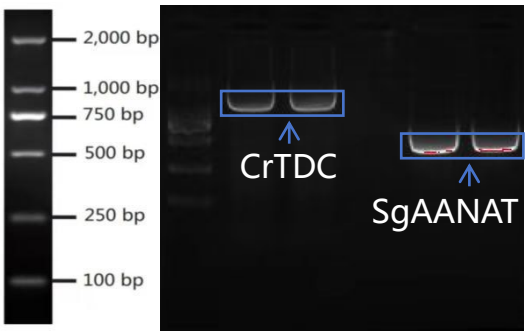
4. Amplification of CrTDC fragments by PCR

PCR system (50μL)	
2×Mix	25μl
CrTDC-F	2μl
CrTDC-R	2μl
template	50ng
DDW	to 50μl

PCR		
98℃	5min	
98℃	30s	×30
59℃	30s	
72℃	90s	
72℃	5min	
25℃	∞	

3.cut gel recovery

The obtained correct bands were cut and recovered, and the PCR products were tested by agarose gel electrophoresis. The results are as follows：



4.connection

Three fragments are connected using the Gibsion assembly method as follows：

Get the pSB1c-SgAANAT-CrTDC plasmid

Gibson system	
0.02×1500bp	30ng
0.02×562bp	11.24ng
0.02×2562bp	51.24ng
2×c115 Mix	5μl
DDW	to 10μl

Gibson.	
50° C	15min
4° C	∞

5.Chemical transformation

Using the chemotaxis method, the ligation product was transformed into DH5α receptor cells and then spread on LB plates containing Chloramphenicol resistance and incubated overnight at 37°C in inverted mode.

6.induction

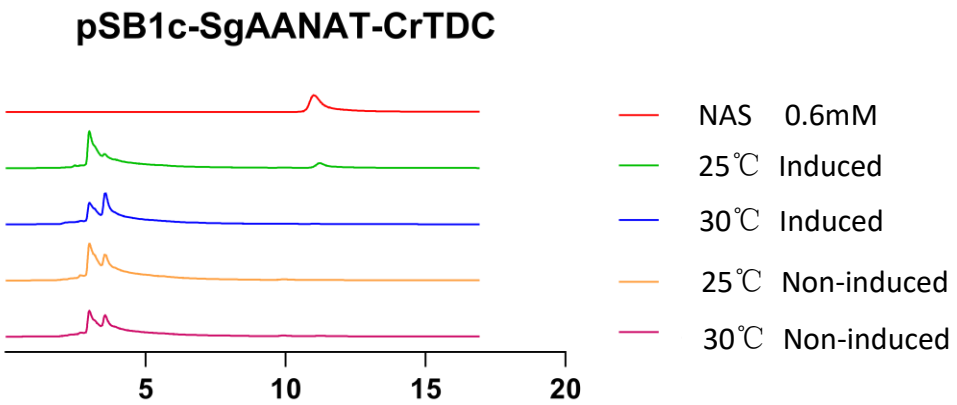
Single clones with correct P results were inoculated into the liquid LB with 0.1% chloramphenicol and cultured for 12h after induction. The induction system is as follows：

Induction system	
ZY	4.8ml
50×M	100μl
5052	100μl
Ara	50μl
MgSO4	10μl
1000×	10μl
菌液	50μl
Chl	5μl

7.Whole cell catalysis

After induction for 16 h, the OD value of the bacterial body in the test tube was tested. 6OD bacteria were taken into a 2ml EP tube and 200μl of the system was added to 50mM Tris-HCL with PH=7.5 and 1mM 5-HTP for whole cell catalysis. After 12 h, samples were taken and HPLC was tested

8.HPLC check



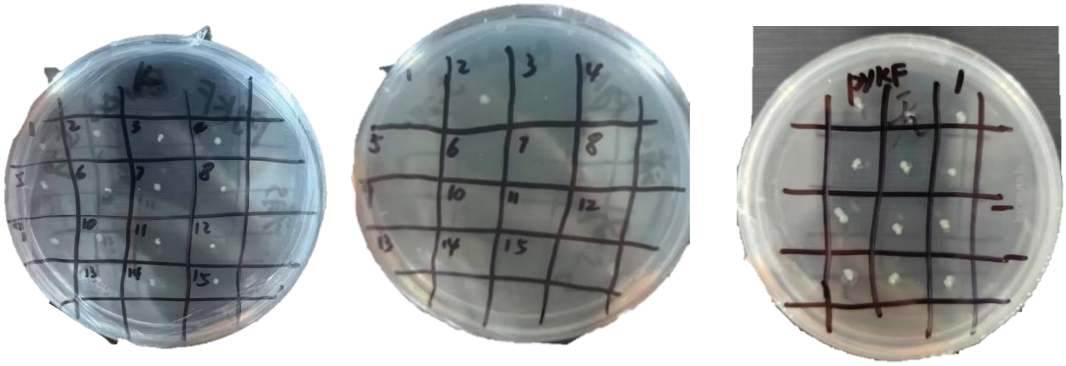
As shown in the figure, strain BW25113/pSB1c-SgAANAT-CrTDC can synthesize NAS from 5-HTP, and the effect is better at 25℃

(B) Plasmid Curing

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

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

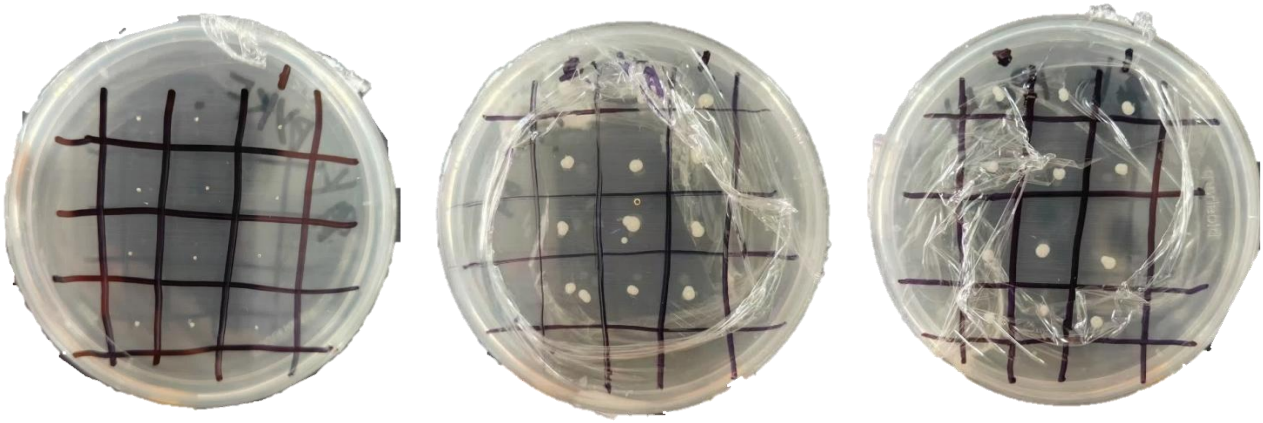
Results: The third curing attempt was successful.



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

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

Results: Curing was successful, and the strain was preserved.



3.Curing of the s-resistant pTarget plasmid from BW25113ΔtrpR ΔtnaAB Δppc

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

Results: The first two curing attempts failed; a third attempt was planned.

(C) Preparation for Knockout: Preparation of Insufficient pTarget-mtr, pTarget-tyrA, pTarget-pykA, pTarget-pykF Plasmids

1.Inoculation and plasmid extraction.

Plasmid concentrations:

pTarget-mtr: 159.241 ng/μL

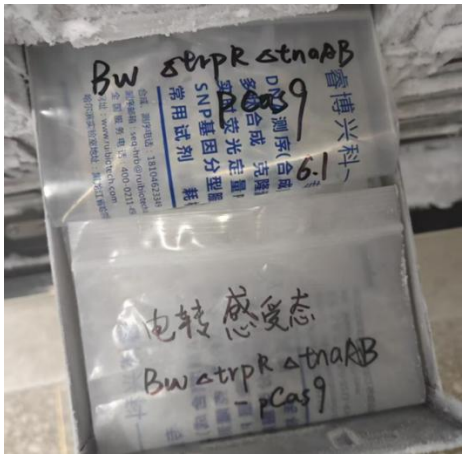
pTarget-ppc: 214.878 ng/μL

pTarget-pykA: 132.604 ng/μL

pTarget-pheA: 203.963 ng/μL

pTarget-zwf: 185.739 ng/Ml

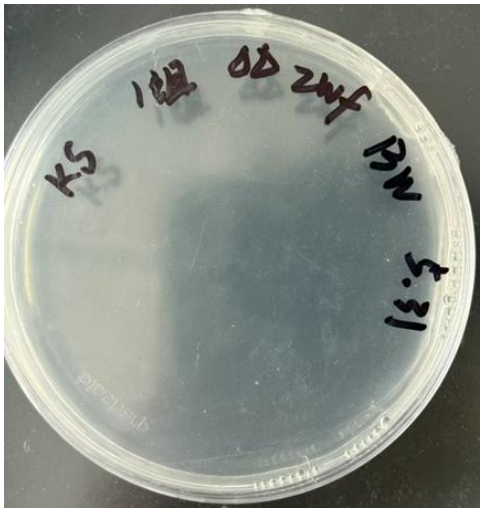
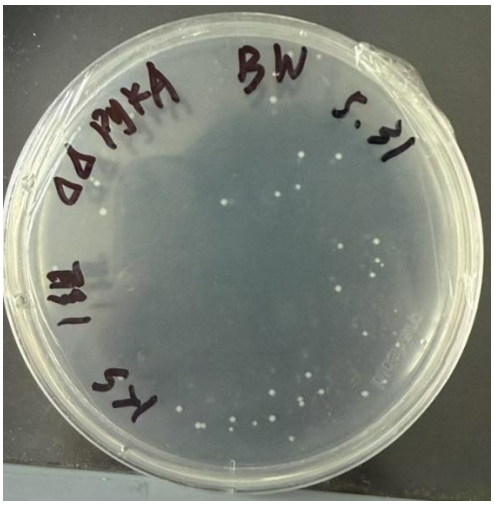
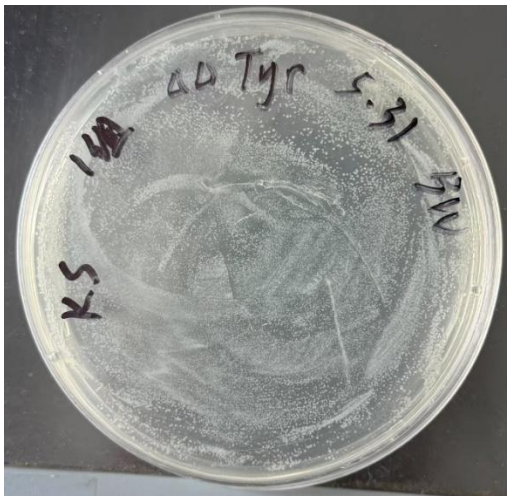
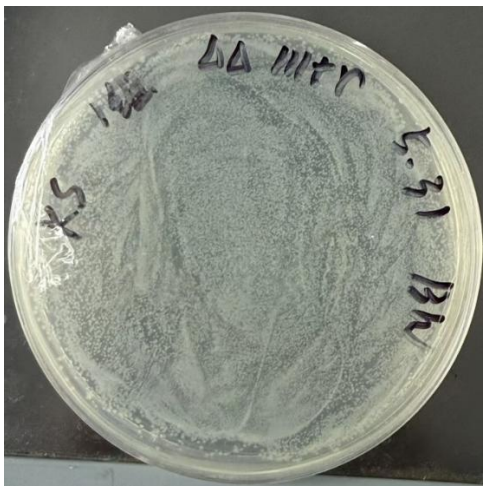
Preparation of insufficient electrotransformation-competent cells of BW25113ΔtrpR ΔtnaAB –pCas9.



(D) Knockout of zwf/pykA/mtr/tyrA Genes

1.Electrotransformation of zwf/pykA/mtr/tyrA genes and plating.

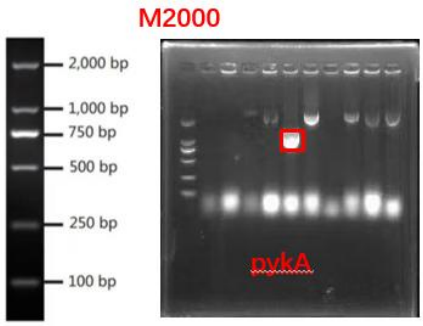
To improve transformation efficiency, 500 ng of targeting fragment and 1000 ng of pTarget plasmid were added.
Results: No colonies grew for zwf; colonies grew for pykA/mtr/tyrA.

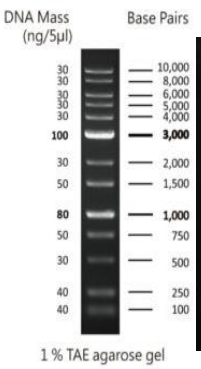





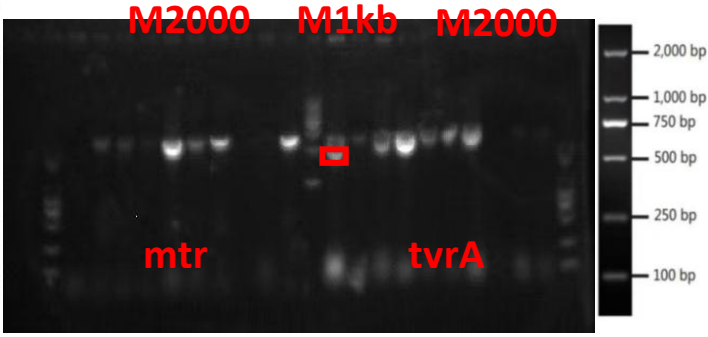
2.Colony PCR Verification for pykA/mtr/tyrA Knockout.

Results: pykA/tyrA knockout was successful with a positive rate of 10%; mtr knockout failed.

Analysis: Multiple knockout attempts for zwf/pheA/mtr genes failed, which was suspected to be due to low specificity of the 20 bp fragment on the pTarget plasmid.







Week2: 6.8-6.14:

（A）Functional validation of co-transfection and mixed bacteria of pYB1a-BH4 and pSB1c-SgAANAT-CrTDC

1.Chemical transformation

The pYB1a-BH4 plasmid was introduced into the Bw25113△trpR△tnaAB strain, while the pSB1c-SgAANAT-CrTDC plasmid was transferred to the same bacterial strain. Both plasmids were co-transfected into the Bw25113△trpR△tnaAB strain. After plate coating and bacterial inoculation, the cultures were incubated for 12 hours.

2.induction

The Bw25113△trpR△tnaAB/pYB1a-BH4 was inoculated into 5ml LB liquid medium containing 0.1% ampicillin. Bw25113/pSB1c-SgAANAT-CrTDC was inoculated into 5ml LB liquid medium containing 0.1% chloramphenicol.

The Bw25113△trpR△tnaAB/pYB1a-BH4/pSB1c-SgAANAT-CrTDC was inoculated into 5ml LB liquid medium containing 0.1% ampicillin and 0.1% chloramphenicol.

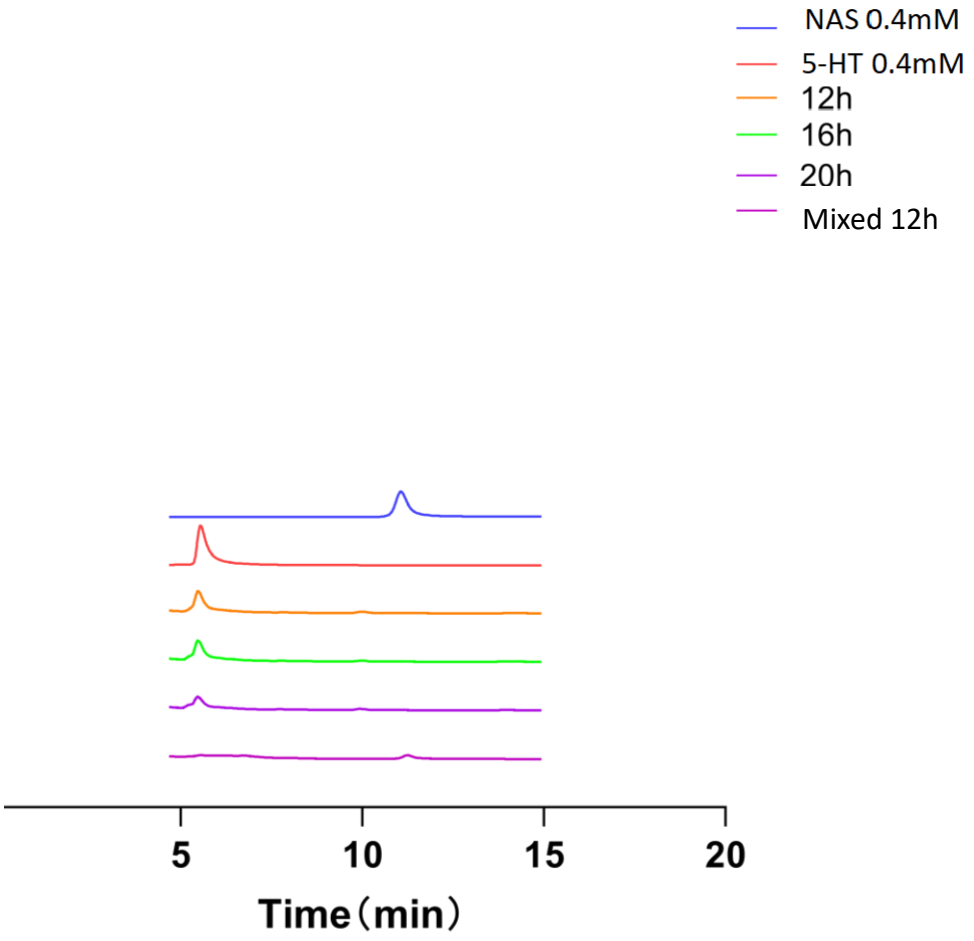
After 12h of cultivation, the induction system is as follows:

Induction system	
ZY	4.8ml
50×M	100μl
5052	100μl
Ara	50μl
$MgSO_4$	10μl
1000×	10μl
bacteria solution	50μl
Amp/Str	5μl

3.Whole cell catalysis

After induction for 16 hours, the OD value of the bacterial cells in the test tube was tested. Take 6OD pYB1a-BH4 and pSB1c-SgAANAT-CrTDC to co-transmute into a 2ml EP tube. After centrifugation at 4200rpm for 10 minutes, discard the supernatant, add 200μl of 50mM Tris-HCl with pH=7.5 and 1mM L-trp, and perform whole cell catalysis of 200μl system. Add 6 OD Bw25113△trpR△tnaAB/pYB1a-BH4 to a 2 mL EP tube. Centrifuge at 4200rpm for 10 minutes, then discard the supernatant. Add 200 μl of 50 mM Tris-HCl (pH 7.5) and 1 mM L-trp, mix by pipetting, then transfer to 6 OD Bw25113/pSB1c-SgAANAT-CrTDC. Perform whole-cell catalysis with 200 μl of the system. The sample was taken after 12h and HPLC was tested.

4.HPLC check



As shown in the figure, strain Bw25113△trpR△tnaAB/pYB1a-BH4/pSB1c-SgAANAT-CrTDC could not realize the synthesis from L-trp to NAS, but BW25113/pYB1a-BH4 and Bw25113/pSB1c-SgAANAT-CrTDC mixed bacteria could realize the synthesis from L-trp to NAS.

（B） Plasmid Curing

1.Curing of plasmids from BW25113ΔtrpR ΔtnaAB ΔpykA.

Results: Both s-resistance and k-resistance were eliminated; the strain was preserved.

2.Curing of the s-resistant pTarget plasmid from BW25113ΔtrpR ΔtnaAB Δppc

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

Results: Five curing attempts failed.

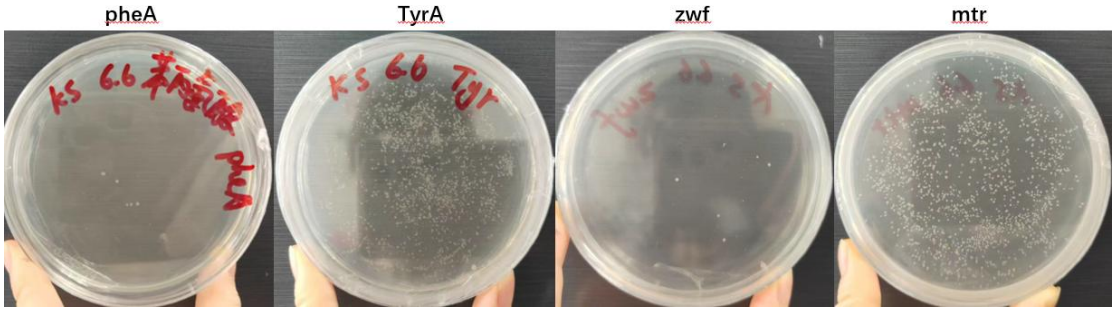
Analysis: It was suspected that the cleavage site of the pTarget plasmid was mutated, preventing the Cas9 protein from recognizing the sequence for cleavage. The pTarget-ppc plasmid was planned to be sent for sequencing verification.

（C） Knockout of zwf/pheA/mtr/tyrA Genes

To improve transformation efficiency, 500 ng of targeting fragment and 1000 ng of pTarget plasmid were added.

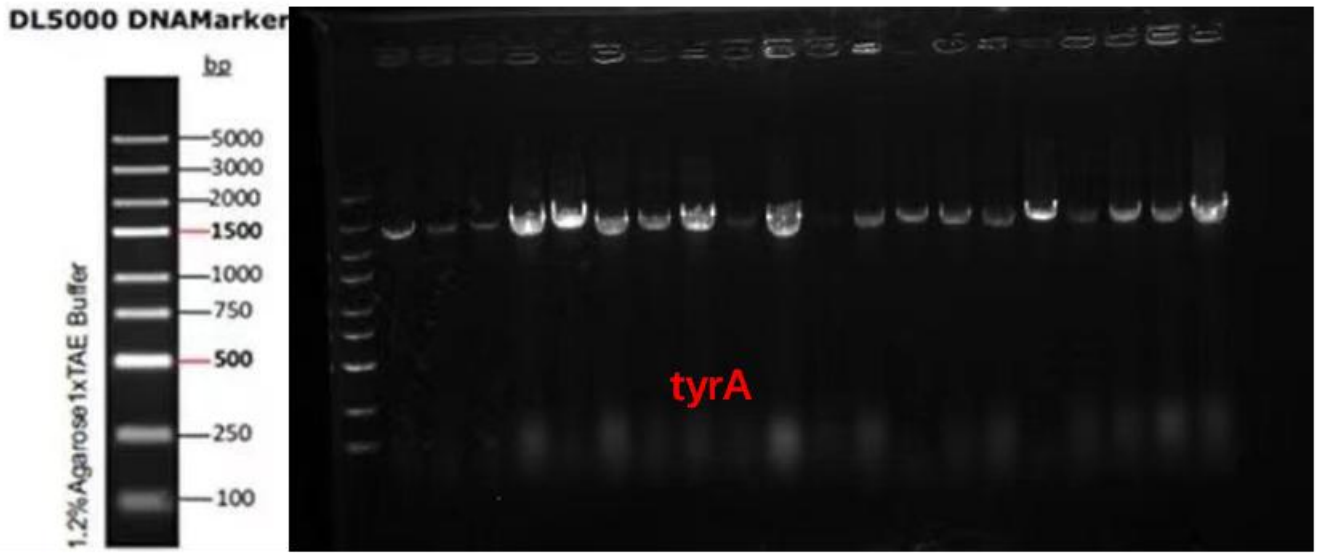
1.Prepare 100 mL of 0.5 mM phenylalanine, and take 50 μL for plating.

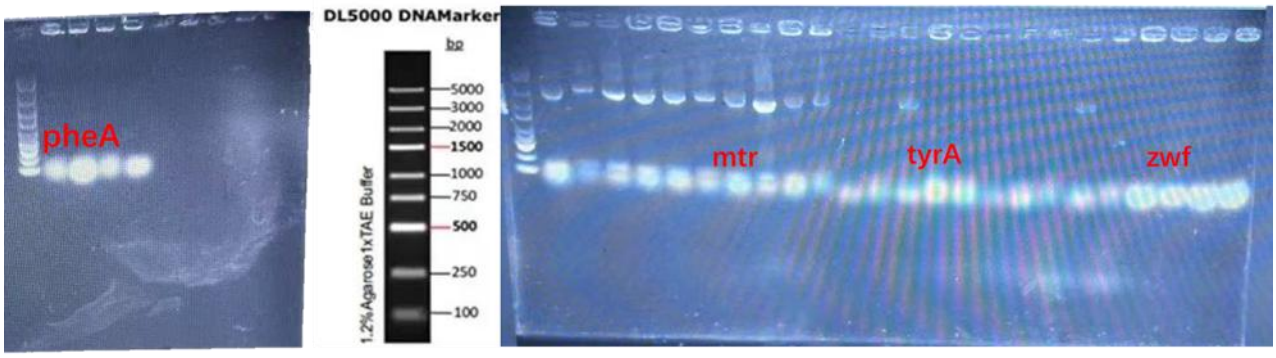
2.Electrotransformation of zwf/pykA/mtr/tyrA genes and plating of diluted bacterial suspension.



3.Colony PCR Verification for pheA/tyrA/zwf/mtr Knockout.

Results: All knockouts failed





4.Preparation for Next Week's Gene Knockout: Preparation of insufficient pTarget-mtr plasmid and zwf targeting fragment

Inoculation and plasmid extraction.

Concentrations:

pTarget-mtr: 115.557 ng/μL

zwf targeting fragment: 220.385 ng/Ml

Week3: 6.15-6.21:

(A)Functional validation of co-transfection of pSB1c-SgAANAT-CrTDC and pYB1a-AtCOMT***

1.Chemical transformation

pYB1a-AtCOMT*** and pSB1c-SgAANAT-CrTDC were co-transfected into the Bw25113 strain and plated.

2.induction

The Bw25113/pYB1a-AtCOMT*****/pSB1c-SgAANAT-CrTDC strain was inoculated into 5 mL of LB liquid medium containing 0.1% ampicillin and 0.1% chloramphenicol. After 12 hours of incubation, induction was performed using the following induction system：

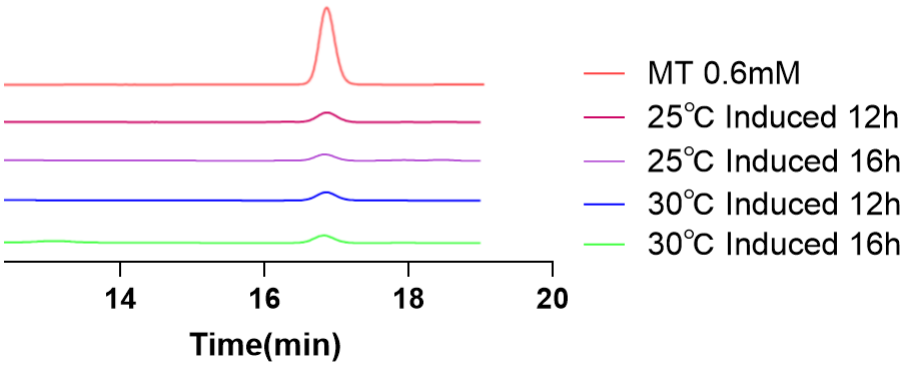
Induction system	
ZY	4.8ml
50×M	100μl
5052	100μl
Ara	50μl
$MgSO_4$	10μl
1000×	10μl
bacteria solution	50μl
Amp	5μl
Chi	5μl

3.Whole cell catalysis

After induction for 16 hours, the OD value of the bacterial cells in the test tube was tested. 6OD

was transferred to a 2ml EP tube, and centrifuged at 4200rpm for 10 minutes. After discarding the supernatant, 200μl of the system containing PH=7.5, 50mM Tris-HCL and 1mM 5-HTP was added for whole cell catalysis. After 12 hours, samples were taken and HPLC was used for detection.

4.HPLC check



As shown in the figure, strain Bw25113/pSB1c-SgAANAT-CrTDC/pYB1a-AtCOMT*** can synthesize MT from 5-HTP, but the yield is low. It is speculated that AtCOMT enzyme activity is not high.

（B）Construction of tyrA Targeting Fragment

Concentration: 99.396 ng/μL

（C）Construction of New ptarget-pheA, ptarget-zwf, ptarget-tyrA, ptarget-mtr Plasmids

1.Introduction of sgRNA by PCR.
2.Purification and digestion of PCR products.
3.Transformation and plating.
4.Shaking culture and plasmid extraction.
Plasmid concentrations:
ptarget-pheA: 107.985 ng/μL
ptarget-zwf: 156.487 ng/μL
ptarget-tyrA: 159.357 ng/μL
ptarget-mtr: 164.142 ng/μL

5.Sequencing.
Results: The sequencing result of ptarget-zwf was correct; double peaks and polyG were observed in ptarget-pheA and ptarget-tyrA.
Analysis: It was suspected that the sequencing primers had multiple binding sites on the template.

生产编号	样品名称	测序引物	样品类型	产品信息	完成情况	样品对应号	结果	备注
YP34999740	PT-PHEA	pt-pheA-cx-F	质粒	PT-PHEA.pt-pheA-cx-F.YP34999740.C02.ab1	本次完成		报告成功	polyG
YP34999741	PT-PHEA	pt-pheA-cx-R	质粒	PT-PHEA.pt-pheA-cx-R.YP34999741.C03.ab1	本次完成		报告成功	双峰
YP34999742	PT-ZWF	pt-zwf-cx-F	质粒	PT-ZWF.pt-zwf-cx-F.YP34999742.C04.ab1	本次完成		报告成功	
YP34999743	PT-ZWF	pt-zwf-cx-R	质粒	PT-ZWF.pt-zwf-cx-R.YP34999743.C05.ab1	本次完成		报告成功	
YP34999744	PT-TYRA	pt-tyrA-cx-F	质粒	PT-TYRA.pt-tyrA-cx-F.YP34999744.C06.ab1	本次完成		报告成功	双峰
YP34999745	PT-TYRA	pt-tyrA-cx-R	质粒	PT-TYRA.pt-tyrA-cx-R.YP34999745.C07.ab1	本次完成		报告成功	双峰

Week 4: 6.22-6.28:

(A)Functional validation of mixed strains pYB1a-BH4 and pSB1c-SgAANAT-CrTDC with pYB1a-AtCOMT***

1.Chemical transformation

pYB1a-BH4 was transferred into the Bw25113△trpR△tnaAB strain, pSB1c-SgAANAT-CrTDC was transferred into the Bw25113 strain, and pYB1a-AtCOMT*** was transferred into the Bw25113 strain, respectively, and the plates were coated

2.induction

The Bw25113△trpR△tnaAB/pYB1a-BH4 was inoculated into 5ml LB liquid medium containing 0.1% ampicillin

Bw25113/pSB1c-SgAANAT-CrTDC was inoculated into 5ml LB liquid medium containing 0.1% chloramphenicol

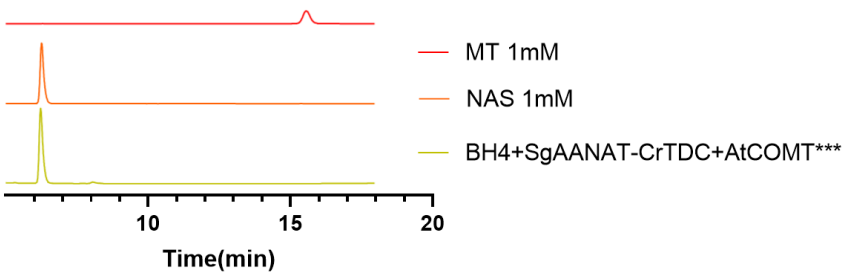
After inoculating Bw25113/pYB1a-AtCOMT*** into 5ml LB liquid medium containing 0.1% ampicillin for 12h, the induction was carried out after 16h. The induction system is as follows：

Induction system	
ZY	4.8ml
50×M	100μl
5052	100μl
Ara	50μl
$MgSO_4$	10μl
1000×	10μl
bacteria solution	50μl
Amp	5μl
Chi	5μl

3.Whole cell catalysis

Add 6OD Bw25113△trpR△tnaAB/pYB1a-BH4 to 2ml EP tubes. Centrifuge at 4200rpm for 10 minutes, then discard the supernatant. Add 200μl of 50mM Tris-HCl (pH 7.5) containing 1mM L-trp, followed by a vigorous beating and mixing. Transfer the mixture to 6OD Bw25113/pSB1c-SgAANAT-CrTDC, then transfer it again to 6OD Bw25113/pYB1a-AtCOMT*** for 200μl full-cell catalysis. After 12h incubation, collect samples for HPLC analysis.

4.HPLC check



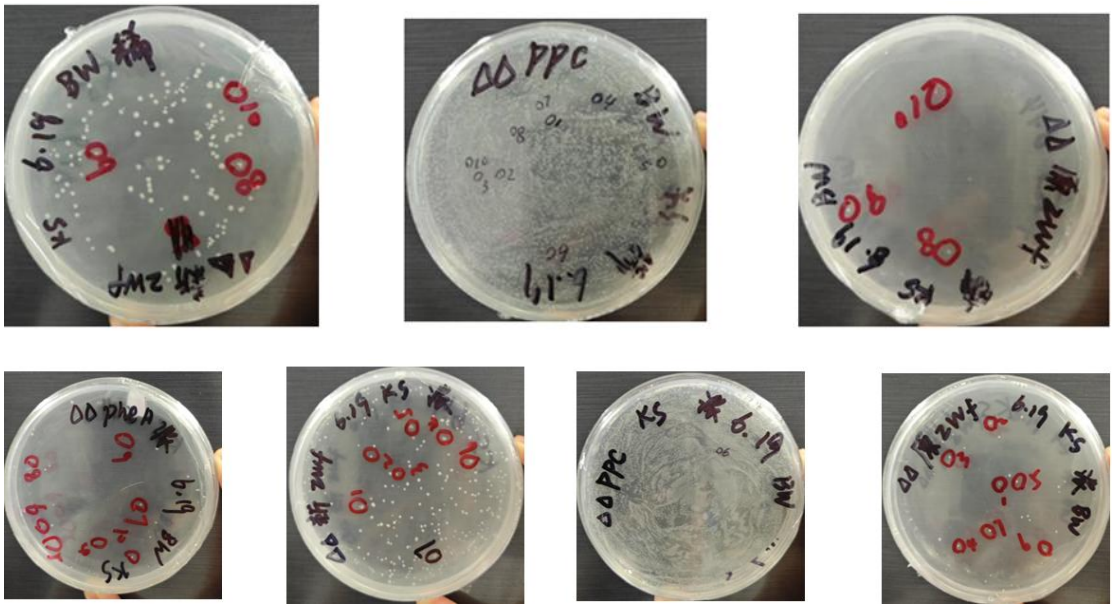
As can be seen from the figure, NAS accumulates in large quantities in the pathway, and the expected synthesis of MT from L-trp does not occur As can be seen from the previous content, when BW25113/pYB1a-BH4 and Bw25113/pSB1c-SgAANAT-CrTDC are mixed, the synthesis from L-trp to NAS can be achieved, and the enzymatic activity of AtCOMT is not high. Therefore, we speculate that the pathway cannot be carried out normally due to the activity problem of AtCOMT enzyme, so we choose to carry out targeted evolution on it.

(B) Knockout of New zwf/Original zwf/pheA/ppc Genes

1.Electrotransformation of new zwf/original zwf/pheA/ppc genes and plating of diluted bacterial suspension.

To improve transformation efficiency, 500 ng of targeting fragment and 1000 ng of pTarget plasmid were added.

2.Prepare 100 mL of 0.5 mM phenylalanine, and take 50 μL for plating.



3.Colony PCR Verification for New zwf/Original zwf/pheA/ppc Gene Knockout.

Results: pheA knockout was successful.

(C) Preparation for Fermentation

1.Preparation of chemically competent cells of triple-knockout strains.
2.Transformation of plasmids into competent cells with partial genes knocked out.

Chassis Strain	Transformed Plasmid
BW ΔtrpR ΔtnaAB ΔptsG	PBR322-trp
BW ΔtrpR ΔtnaAB ΔpykA	PBR322-trp
BW ΔtrpR ΔtnaAB ΔpykF	PBR322-trp
BW	
BW	Pya-P23119-trpEDCBA
BW	Pya-P23119-trpES40FDCBA
BW	PBR322-trp
BW ΔtrpR	
BW ΔtrpR	Pya-P23119-trpEDCBA
BW ΔtrpR	Pya-P23119-trpES40FDCBA
BW ΔtrpR	PBR322-trp
BW ΔtrpR ΔtnaAB	
BW ΔtrpR ΔtnaAB	Pya-P23119-trpEDCBA
BW ΔtrpR ΔtnaAB	Pya-P23119-trpES40FDCBA
BW ΔtrpR ΔtnaAB	PBR322-trp

Last week, we initially verified the basic function of the biosensor. This week, our goal was to conduct more rigorous and in-depth quantitative characterization to lay a solid foundation for subsequent high-throughput screening. The specific objectives included:

1.Quantification: Accurately evaluate the basal leakage expression level of the sensor in the absence of signal (tryptophan).

2.Validation of Screening Window: Verify that the sensor can provide a sufficiently large growth difference in the presence and absence of tryptophan, thereby determining the optimal screening conditions.

(D) In-depth Characterization: Quantifying Sensor Leakiness

An excellent biosensor must have low "background noise" (i.e., low leakage expression), which is a prerequisite for ensuring the accuracy of screening results. Last week, we initially observed the leakage phenomenon; this week, we designed a more precise experiment to quantify its specific impact under different selection pressures.

1.Solution Preparation

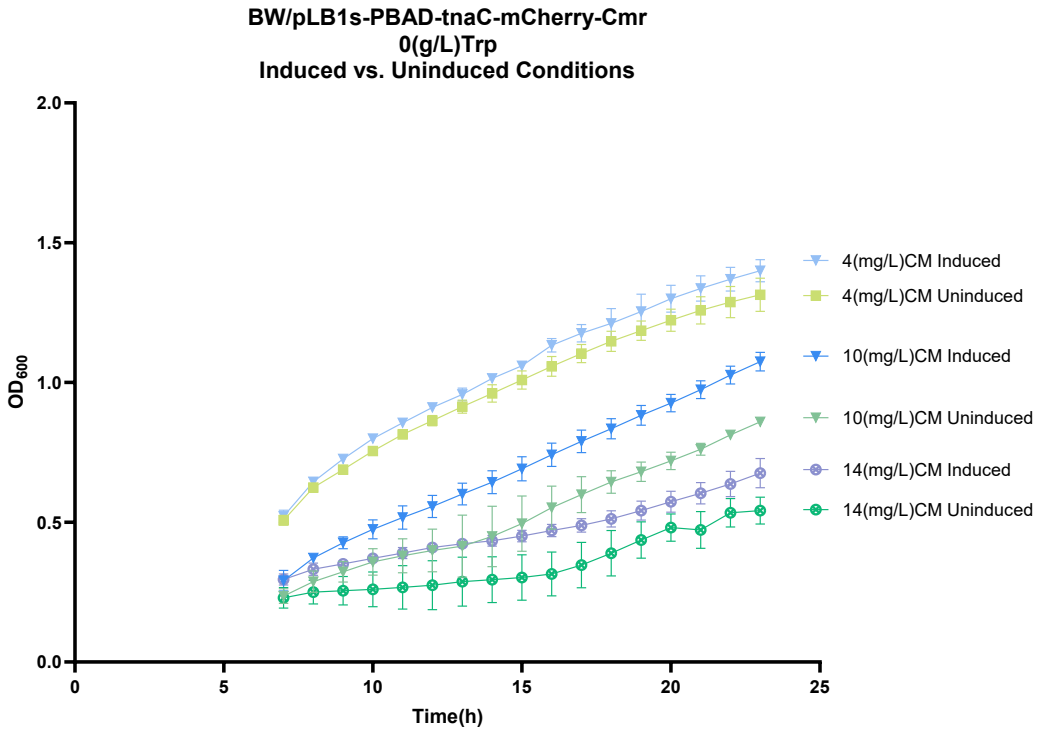
Prepare M9-Y solution according to the above formula.

2.Addition of Inducer and Chloramphenicol.No tryptophan was added.

Set up two control groups: one without arabinose addition, and the other with 1 mg/L arabinose addition. Each group included experimental subgroups with different chloramphenicol concentrations (4, 10, 14 mg/L) to observe the survival of the strain due to leakage expression under different selection pressures.

3.Growth Curve Plotting

Inoculate the strain into a 96-well plate and continuously monitor the 24-hour growth curve (OD_{600}) using a microplate reader.



Result: The growth curve of the induced group was slightly higher than that of the uninduced group, which confirmed that our PBAD promoter system has a low level of leakage expression. However, a key finding was that with the increase of CM concentration, this weak growth advantage caused by leakage was effectively inhibited. This indicates that although leakage exists, it is controllable. By applying sufficient selection pressure, we can effectively eliminate background noise and ensure that only real positive signals can survive.

(E) Validating the Sensor's Dynamic Range and "Signal" Strength

The "signal" of the sensor refers to the growth advantage brought by the presence of tryptophan. We need to prove that this "signal" is strong enough to be clearly distinguished from the "noise"

(leakage), thereby forming a reliable screening window.

1.Solution Preparation

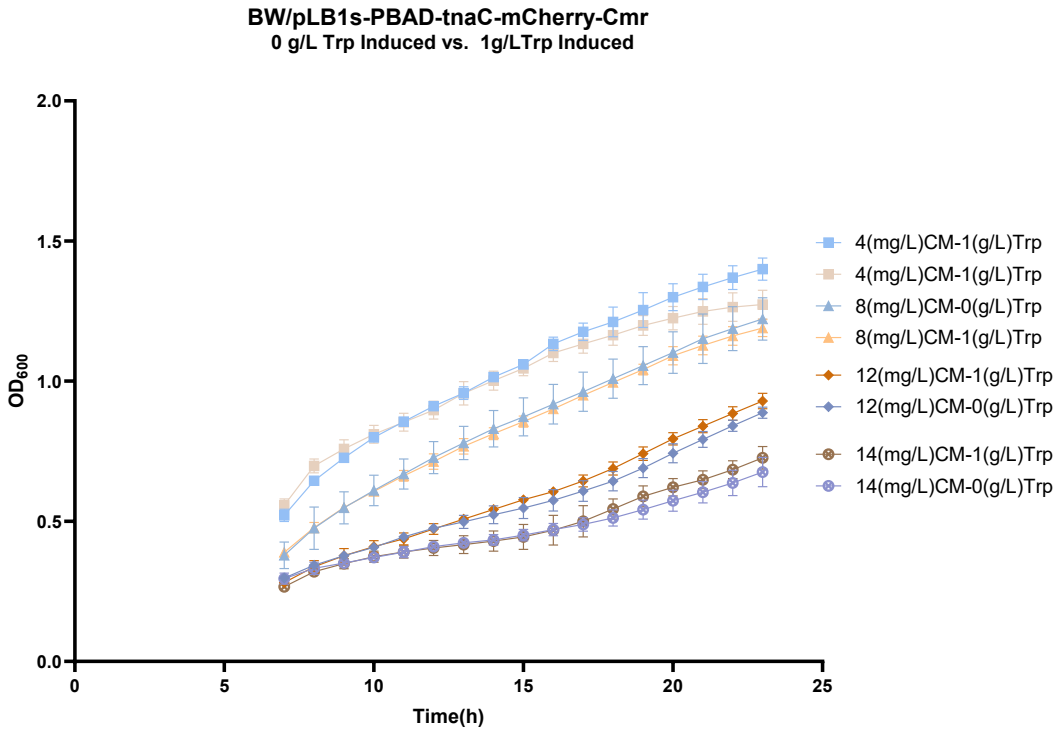
Prepare M9-Y solution using the above formula.

2.Addition of Inducer, Chloramphenicol, and Tryptophan

Add arabinose to a final concentration of 1 mg/L. Set up two experimental groups: one without tryptophan addition, and the other with 1 g/L tryptophan addition. Each group included experimental subgroups with different chloramphenicol (CM) concentrations (4, 8, 12, 14 mg/L). Each concentration was tested in triplicate.

3.Growth Curve Plotting

Inoculate the strain into a 96-well plate and continuously monitor the 24-hour growth curve (OD_{600}) using a microplate reader.



Result: At low CM concentrations, the strain could grow regardless of the presence or absence of tryptophan. However, with the increase of CM concentration (> 8 mg/L), the two growth curves began to separate significantly: the growth of the Trp-free group was gradually inhibited, while the Trp-containing group could maintain robust growth. This revealed a broad and effective "working window" for us. In the CM concentration range of 12-14 mg/L, the growth difference between the presence and absence of tryptophan reached the maximum. This is the ideal condition for future high-throughput screening experiments, as it can most effectively distinguish "positive" from "negative" strains.