The experimental objective in August: Overexpressing individual genes in the shikimic acid pathway to explore whether it can increase tryptophan yield.The yield of the whole-genome mutant strain was tested by fermentation. The metabolic pathway was further optimized, and glnA and yddG genes were overexpressed to test the level of tryptophan synthesis.

Week1: 8.1-8.7:

(A) pSB1c-aroG，pSB1c-xfpK，pSB1c-ppsA，pSB1c-tktA，pSB1c-aroG-tktA-ppsA, pSB1c-aroB，pSB1c-aroD，pSB1c-aroEPlasmid Construction

1.Amplify the vector via PCR

The target vector fragment was obtained via PCR using the following PCR system:

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

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

The PCR products were analyzed by agarose gel electrophoresis, revealing the correct target fragment length of 1572 bp. We obtained the correct target fragment and proceeded with gel extraction.

2.(1)Amplify the aroG fragment via PCR

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

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

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

The PCR products were analyzed by agarose gel electrophoresis, revealing the correct target fragment length of 2938 bp. We obtained the correct target fragment and proceeded with gel extraction.

2.(2)Amplify the xfpK fragment via PCR

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

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

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

The PCR products were analyzed by agarose gel electrophoresis, revealing the correct target fragment length of 2478 bp. We obtained the correct target fragment and proceeded with gel extraction and recovery of the sample.

2.(3)Amplify the ppsA fragment via PCR

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

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

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

The PCR products were analyzed by agarose gel electrophoresis, revealing the correct target fragment length of 4264 bp. We obtained the correct target fragment and proceeded with gel extraction and recovery of the sample.

2.(4)Amplify the tktA fragment via PCR

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

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

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

The PCR products were analyzed by agarose gel electrophoresis, revealing the correct target fragment length of 3877 bp. We obtained the correct target fragment and proceeded with gel extraction and recovery of the sample.

2.(5) Amplify the aroG-tktA-ppsA fragment via PCR

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

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

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

The PCR products were analyzed by agarose gel electrophoresis, revealing the correct target fragment length of 5424 bp. We obtained the correct target fragment and performed gel extraction to recover the sample.

2.(6)Amplify the aroB fragment via PCR

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

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

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

The PCR products were analyzed by agarose gel electrophoresis, revealing the correct target fragment length of 2974 bp. We obtained the correct target fragment and proceeded with gel extraction and recovery of the sample.

2.(7)Amplify the aroD fragment via PCR

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

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

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

The PCR products were analyzed by agarose gel electrophoresis, revealing the correct target fragment length of 2644 bp. We obtained the correct target fragment and proceeded with gel extraction and recovery of the sample.

2.(8)Amplify the aroE fragment via PCR

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

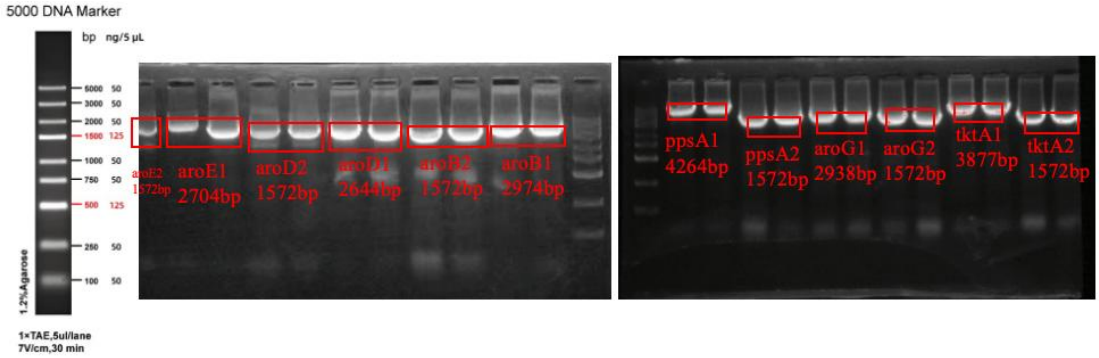
PCR system(50μL)	
2×Mix	25ul
aroE-F	2ul
aroE-R	2ul
Template	50ng
DDW	20ul

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

The PCR products were analyzed by agarose gel electrophoresis, revealing the correct target fragment length of 2704 bp. We obtained the correct target fragment and proceeded with gel extraction and recovery of the sample.

3.Rubber Recycling

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



4.Using the Gibson assembly method to join two fragments

The specific procedure is as follows: Connect using Gibson (C116) to obtain pSB1c-aroG, pSB1c-xfpK, pSB1c-ppsA, pSB1c-tktA, pSB1c-aroG-tktA-ppsA, pSB1c-aroB, pSB1c-aroD, pSB1c-aroE

pSB1c-aroG system	
0.02×1572bp	ng
0.04×2938bp	ng
2×c115 Mix	5ul
DDW	to10ul

pSB1c-xfpK system	
0.02×1572bp	ng
0.04×2478bp	ng
2×c115 Mix	5ul
DDW	to10ul

pSB1c-ppsA system	
0.02×1572bp	ng
0.04×4264bp	ng
2×c115 Mix	5ul
DDW	to10ul

pSB1c-tktA system	
0.02×1572bp	ng
0.04×3877bp	ng
2×c115 Mix	5ul
DDW	to10ul

pSB1c-aroE system	
0.02×1572bp	ng
0.04×2704bp	ng
2×c115 Mix	5ul
DDW	to10ul

Gibson	
50℃	30min
4℃	∞

pSB1c-aroG-tktA-ppsA	
0.02×1572bp	ng
0.04×5424bp	ng
2×c115 Mix	5ul
DDW	to10ul

pSB1c-aroB system	
0.02×1572bp	ng
0.04×2938bp	ng
2×c115 Mix	5ul
DDW	to10ul

pSB1c-aroD system	
0.02×1572bp	ng
0.04×2644bp	ng
2×c115 Mix	5ul
DDW	to10ul

5.Chemical Conversion

Using chemical transformation, the ligation products were transferred into DH5α competent cells and spread onto LB plates containing chloramphenicol resistance. After overnight incubation at 37°C, the expected resistant colonies grew on the plates.

6.Selective Cloning Inoculation

After overnight incubation at 37°C, pick one colony from the plate. Inoculate the single colony into liquid medium and incubate at 37°C for 12 hours.

7.Plasmid extraction

Sequencing results for pSB1c-aroG, pSB1c-xfpK, pSB1c-ppsA, pSB1c-tktA, pSB1c-aroG-tktA-ppsA, pSB1c-aroB, pSB1c-aroD, and pSB1c-aroE were correct.

（B）High ATCOMT enzyme activity strains were selected in one round

1.Perform error-prone PCR

PCR system (Mg^{2+}：Mn^{2+}=10：1）	
2×Mix Green Master	25μl
Mg^{2+}(10mM)	1μl
Mn^{2+}(1mM)	1μl
AtCOMT-R	2μl
AtCOMT-F	2μl
Templete	50ng
DDW	to 50μl

2.Product purification

The products of error-prone PCR were purified

3.connection

The plasmid pYB1a-AtCOMT* 50 tube was constructed by golden gate connection. The connection system is as follows:

BsaI	0.5μL
rCutSmart Buffer	1μL
T4 DNA Ligase	0.2μL
10×Ligase Buffer	1μL
ddH2O	to 10μL
PYB1a-ccdB	150ng
AtCOMT***	150ng

37℃	30 min
37℃	3 min
16℃	3 min
16℃	20 min
37℃	20 min
55℃	15 min
80℃	15min

4.Transformation and transduction

The 50 tubes containing the plasmid transformation were transferred into DH5α. After extracting the mixed bacterial plasmids, they were transferred into the cysteine-deficient strain BWΔCysE-PLB1s-Cys3-Cys4. 50μL was then transferred to M9 medium with added Cys for 30℃ days of transition. The transfer system is as follows:

5ml Transitional system	
M9	1mL
Amp,Str	各 5μL
20%glycerol	0.5ml
MgSO4	10μL
CaCl2	0.5μL

Ara	50μL
Cys	0.2mM
H2O	3.5ml

5.selection

250μL of bacterial fluid was taken from the transition system and transferred to the screening system for screening. The screening system is as follows:

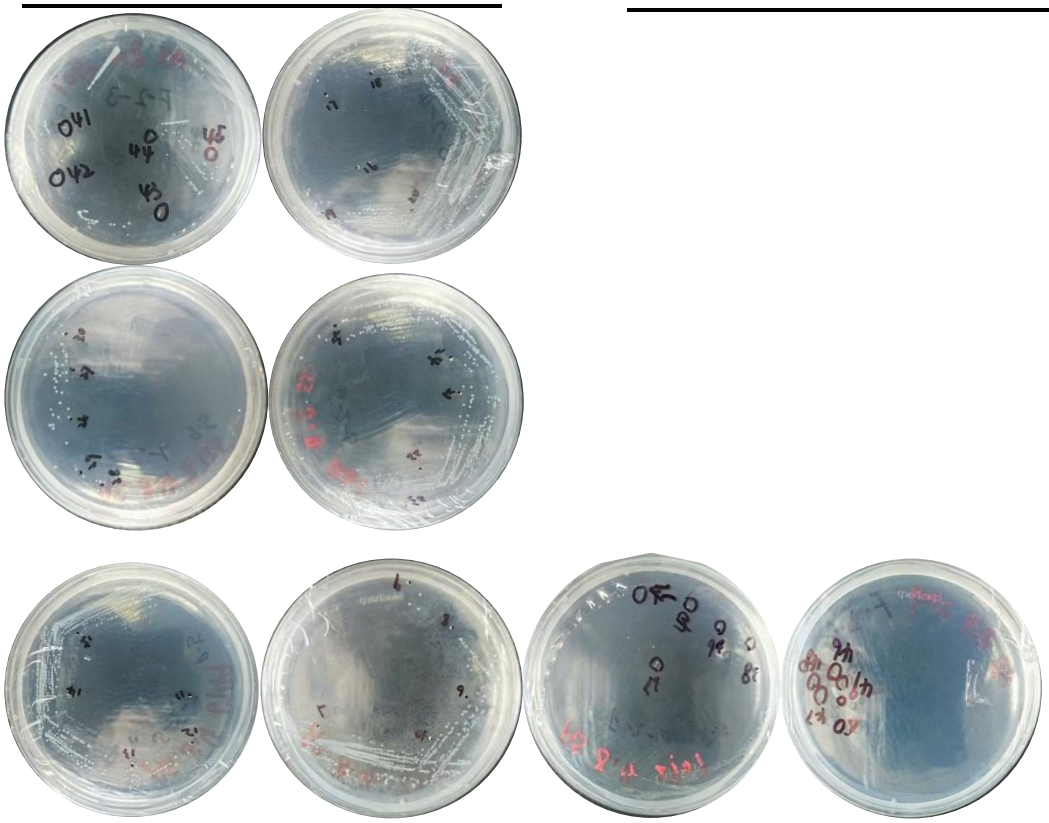
5ml selection system	
M9	1mL
20%glycerol	0.5ml
MgSO4	10 μL
CaCl2	0.5 μL
Ara	50 μL
bacteria solution	250μL
H2O	3.5ml
SAM	100uL（2mM）
NAS	100uL（1mM）

6.Obtain a library of single-cloned mutants

Cultivate the bacteria in the screening system for 16h. After the bacterial liquid becomes slightly turbid, 250μL of the bacterial liquid is drawn into the screening system. Repeat the process until the bacterial liquid becomes slightly turbid again. Then make a plate streak and pick a single colony for inoculation.

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

PCR		
98℃	5min	
98℃	30s	×30
58℃	30s	
72℃	131s	
72℃	5min	
25℃	∞	



(C) Construction of pSB1c-P23119-yddG plasmid：

1. Amplification of the vector by PCR

Target vector fragments were obtained by PCR with the RCR system as follows.

The PCR product was detected by agarose gel electrophoresis and the correct target fragment length was 4362 bp. We obtained the correct target fragment and the samples were cut and recovered.

2.The yddG fragment was amplified by PCR

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

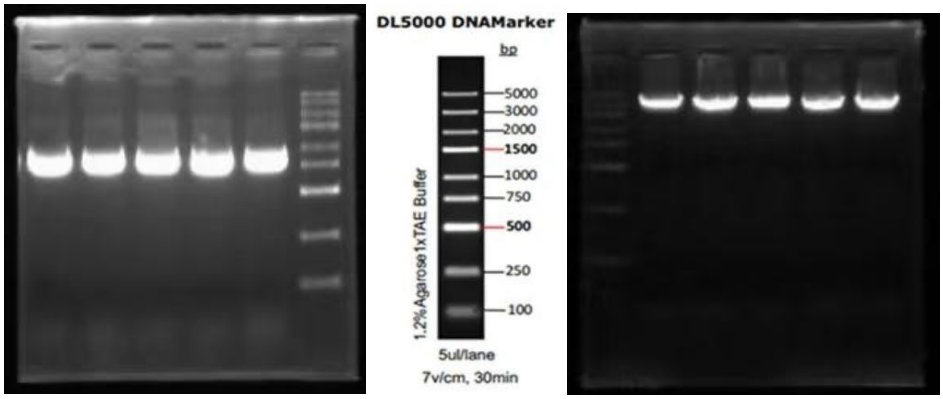
PCR system (50μL)	
2×Mix	25ul
yddG-F	2ul
yddG-R	2ul
Template	20ng
DDW	20ul

PCR		
98℃	5min	
98℃	30s	×30
58℃	30s	
72℃	28s	
72℃	5min	
25℃	∞	

The PCR product was detected by agarose gel electrophoresis, and the correct target fragment length was 902 bp. We obtained the correct target fragment and the samples were cut and recovered.

3. Gel extraction

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



4. Use Gibsion assembly method to join two fragments

The specific operation is as follows:

The plasmid pSB1c-P23119-yddG was obtained using Gibson (C116) ligation with 4362 bp of vector and 902 bp of yddG.

Gibson system	
0.02×4362bp	ng
0.02×902bp	ng
2×c116 Mix	5ul
DDW	To 10ul

Gibson	
50℃	15min
4℃	∞

5. Chemical transformation

The ligation products were chemically transformed into DH5α competent cells and plated on LB plates containing Cmr resistance and incubated at 37°C overnight.

6.Pick a single clone for inoculation:

After overnight incubation at 37 ° C, the expected resistant single colonies grew on plates. The monoclonal clones were inoculated into liquid medium and incubated for 12 hours at 37 ° C.

7. Plasmid extraction

The results of pSB1c-P23119-yddG sequencing were correct.

(D) Construction of pLB1s-PBAD-glnA-prs plasmid:

1. The vector fragment was obtained by enzyme digestion

XhoI and SpeI in the plasmid map were selected as restriction sites and digested to obtain the vector fragment.

Restriction enzyme digestion system （10ul）	
Template	1ul
XhoI	0.2ul
SpeI	0.2ul
Cutsmart buffer	1ul
DDW	To 10ul

Reaction	
37℃	2h

2.(1) Amplification of glnA fragments by PCR

The target fragments were obtained by PCR with the RCR system as follows.

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

PCR		
98℃	5min	
98℃	30s	×30
56℃	30s	
72℃	44s	
72℃	5min	
25℃	∞	

The PCR product was detected by agarose gel electrophoresis and the correct target fragment length was 1452 bp. We obtained the correct target fragment and the samples were cut and recovered.

2.(2)Amplification of prs fragments by PCR

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

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

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

The PCR product was detected by agarose gel electrophoresis, and the correct target fragment length was 991 bp. We obtained the correct target fragment and the samples were cut and recovered.

3. Gel extraction

The correct bands obtained were cut and recovered, and the PCR products were detected by agarose gel electrophoresis.

4. Use Gibsion assembly method to join three fragments

The pLB1s-PBAD-glnA-prs plasmid was obtained using Gibson (C116) ligation with 3061 bp of vector, 1452 bp of glnA, and 991 bp of prs.

Gibson system	
0.02×4362bp	ng
0.04×902bp	ng
0.04×991bp	ng
2×c116 Mix	5ul
DDW	To 10ul
Gibson	
50℃	30min
4℃	∞

5. Chemical transformation

The ligation products were chemically transformed into DH5α competent cells and plated on LB plates containing Cmr resistance and incubated at 37°C overnight.

6. Pick a single clone for inoculation:

After overnight incubation at 37℃, the expected resistant single colonies grew on plates. The monoclonal clones were inoculated into liquid medium and incubated for 12 hours at 37℃.

7. Plasmid extraction,The pLB1s-PBAD-glnA-prs sequence was correct.

Week2: 8.8-8.14:

(A) Preparation of BWΔtrpRΔtnaABΔpheA Competent Cells

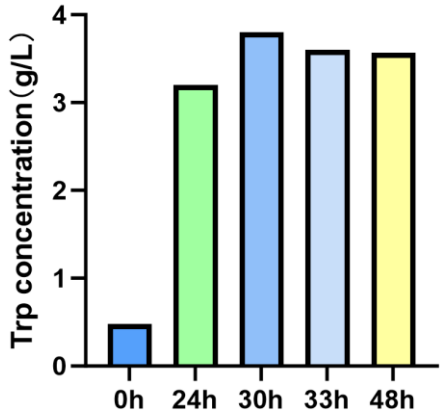
First, streak the original strain and pick a single colony to inoculate into 5 mL LB medium. Incubate overnight at 37°C on a shaking incubator. Transfer 1 mL of the culture to 100 mL fresh LB medium. Shake at 30°C until the OD reaches approximately 0.2, then transfer to a 16°C shaking incubator (pre-cooled) and shake until the OD slightly exceeds 0.4. Ice-bathe for 15 min. Aliquot LB from the conical flask into 4 × 50 mL centrifuge tubes (approximately 24–25 mL per tube). Centrifuge at 4°C and 4000 rpm for 10 min; discard supernatant. Add 1.6 mL of Solution 1 to each tube. After dispersing the cells, combine all tubes into one. Incubate on ice for 30 min, then centrifuge for 10 min. Discard the supernatant. Add 3 mL of Solution 2 to the tube, disperse the cells, and aliquot 100–200 μL into each tube. Store at -80°C.

(B) Fermentation

1.Single-step fermentation: Testing tryptophan yield after introducing the plasmid into the chassis strain

Single transformation of the pBR322-aroG-SerA-trpDCBA plasmid into BWΔtrpRΔtnaABΔpheA achieved a maximum fermentation yield of 3.8 g/L at 30 hours.

Results are as follows:



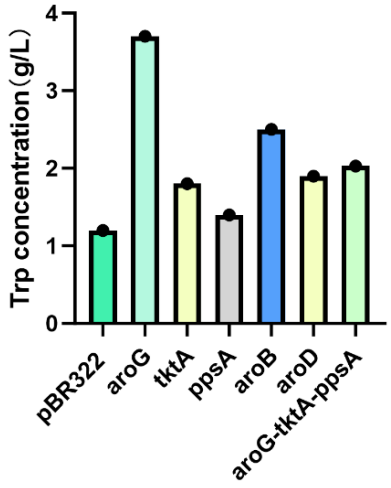
2. Double-forward fermentation: Testing whether individual genes in the shikimic acid pathway can enhance tryptophan yield.

Transform pSB1c-aroG, pSB1c-ppsA, pSB1c-tktA, pSB1c-aroB, pSB1c-aroD, and pSB1c-aroG-tktA-ppsA into BWΔtrpRΔtnaABΔpheA competent cells. Plan to conduct dual-fermentation yield tests.

（C）Liquid-phase testing

Tests were conducted at 0h, 12h, 24h, 30h, 36h, and 48h. Results showed that at 36h fermentation, both double-transformed plasmids yielded higher than the single-transformed pBR322 plasmid. Among them, the highest yield of 3.7g/L was achieved by adding the aroG gene to the enhanced pBR322.

Results are as follows:



(D) Replace the promoter of pLB1s-PBAD-glnA-prs with P23107:

1. Amplify pLB1s - P23107 - glnA - prs by PCR

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

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

PCR		
98℃	5min	
98℃	30s	×30
55℃	30s	
72℃	164s	
72℃	5min	
25℃	∞	

The polymerase chain reaction products were detected by agarose gel electrophoresis, and the correct target fragment with a length of 5439 bp was obtained. We obtained the correct target fragment and recovered the sample by gel extraction.

2. Gel extraction

Excise the correct band obtained for gel extraction. Perform agarose gel electrophoresis on the PCR products.

3. Digestion

Eliminate the PCR template. The reaction system is as follows:

Restriction digestion system (10ul)	
DNA	200ng
rCutsmart buffer	1ul
DpnI	0.2ul
DDW	To 10ul

Restriction digestion reaction	
37℃	2h
55℃	15min
80℃	15min

4. Chemical transformation

Use the chemical transformation method to transform the ligation product into DH5α competent cells, then spread the cells on an LB plate containing Cmr resistance, and incubate overnight at 37°C.

5. Picking and inoculating single colonies

After overnight incubation at 37°C, the expected resistant single colonies grow on the plate. Inoculate the single colonies into the liquid medium and culture them at 37°C for 12 hours.

6. Extract the plasmid and sequence it.

The sequencing result of pLB1s - P23107 - glnA - prs is correct.

(E) Tryptophan synthesis levels of mutant bacteria in the 5th and 10th rounds of fermentation testing:

the tryptophan synthesis levels of 10 bacteria each from the fifth and tenth rounds of whole - genome mutation.

Control group: BWΔtrpRΔtnaAB - pYB1a - P23119 - tnaE^{S40F}DCBA

Experimental group: 10 strains of bacteria each from the fifth and tenth rounds of whole - genome mutation.

1. Chemical transformation to obtain control strains:

Transfer pYB1a - P23119 - tnaE^{S40F}DCBA into the competent cells of BWΔtrpRΔtnaAB as the control group. Spread the cells on LB plates containing Amp and incubate overnight at 37°C. The expected resistant single colonies grow on the plates.

2. Streaking:

Streak the mutant bacteria from the 5th and 10th rounds on plates (LB plates containing Amp, Smr, and Kana resistance) to isolate single clones.

3. Pick single：

clone inoculation: Randomly select 10 single clones each from the streaked plates of the 5th and 10th round mutant bacteria and 1 single clone from the control strain plate, and inoculate them into liquid LB medium and culture at 37°C for 12 hours.

4.Plating:

Use an inoculation loop to dip the bacterial solution three times from the liquid LB medium and spread it on the corresponding resistant LB plates of each strain until the plates are fully covered, and then culture at 37°C for 12 hours.

5. Inoculation into seed medium:

Use 50 ml EP tubes as containers for the seed medium. The system ratio is as follows:

Seed culture medium system (3ml)	
Seed culture medium	2.7ml
Seed glucose	300ul
Antibiotics corresponding to the strain	3ul

crape the colonies on the plate with an inoculating loop and transfer them to the seed medium. Then, incubate them in a shaker at 37°C for 18 hours until the OD value reaches 1 - 1.2.

6. Inoculate into the fermentation medium:

Take 1.2 OD of the bacterial solution into a 10 ml EP tube. Pre - cool the centrifuge at 4 °C, and set the centrifugation program to 4200 rpm for 10 min. In the clean bench, use a 50 ml EP tube as a container to prepare the fermentation medium. The system ratio is as follows:

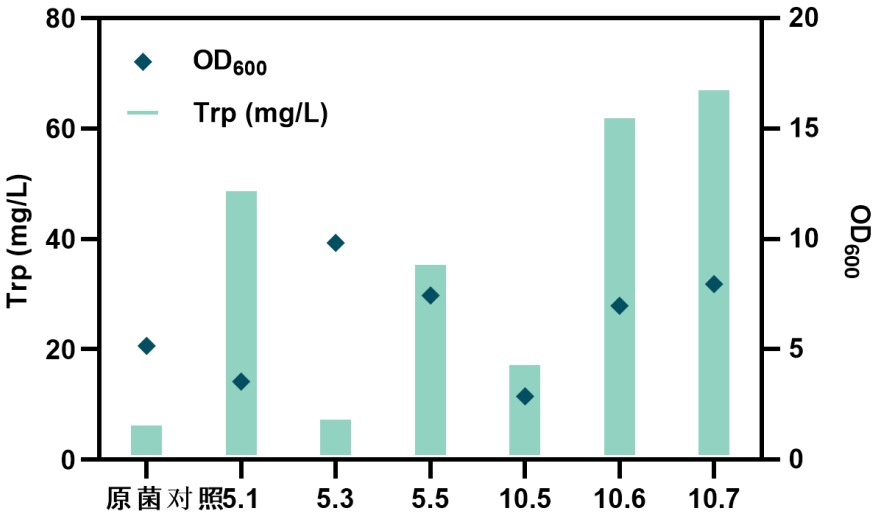
Fermentation culture medium system (3ml)	
Fermentation medium	2.4ml
Seed medium	300ul
Fermentation glucose	300ul
Antibiotics corresponding to the strain	3ul

In the ultra-clean environment, discard the supernatant in the 10 ml EP tube after centrifugation. Pipette 300 μl of the prepared fermentation medium into the centrifuged EP tube, pipette up and down to resuspend the colonies. Pour all the liquid in the EP tube back into the medium, and then place it in a shaker incubator at 37 °C for cultivation.

7. Measure OD values with a spectrophotometer, prepare samples for liquid chromatography:

Take 500 μL of the bacterial solution from each Erlenmeyer flask containing the strains. Among them, take 200 μL of the bacterial solution and measure the OD value with a spectrophotometer. At 28 h, it was judged that the growth of each strain had basically reached the plateau phase through the OD value. The remaining 300 μL of the bacterial solution was used for sample preparation. After centrifugation at 10,000 g for 10 min, the supernatant was aspirated with a syringe, filtered through a membrane, and injected into a small liquid - phase vial.

8. Determination of tryptophan production by high-performance liquid chromatography (HPLC):



The tryptophan standard product showed a peak at 9 minutes, but only 6 strains in the experimental group showed peaks at 9 minutes, and the rest did not show peaks. Since the tryptophan (Trp) production of the original strain control was too low, the three strains with the highest yield, 5.1/10.6/10.7, will be selected for re - fermentation verification. To increase the accuracy of the original strain control, three parallel experiments will be conducted on the control group in the next fermentation.

Week3: 8.15-8.21:

(A)Construction of aroG mutants (pSB1c-p23119-aroGD6G-D7A, pSB1c-p23119-aroGS180F, pSB1c-p23119-aroGS211F):

Investigate whether it is possible to further enhance tryptophan yield based on aroG.

1. Construction of aroG Mutants

Using the pSB1c-p23119-aroG construct as a template, PCR amplification was performed to generate three aroG mutants: pSB1cc-p23119-aroGD6G-D7A, pSB1c-p23119-aroGS180F, pSB1c-p23119-aroGS211F.The PCR system is as follows:

PCR system(50ul)	
2 Mix	25ul
aroGD6G-D7A-F	2ul
aroGD6G-D7A-R	2ul
Template	50ng
DDW	20ul

PCR		
98℃	5min	
98℃	30s	×330
56℃	30s	
72℃	90s	
72℃	5min	
25℃	∞	

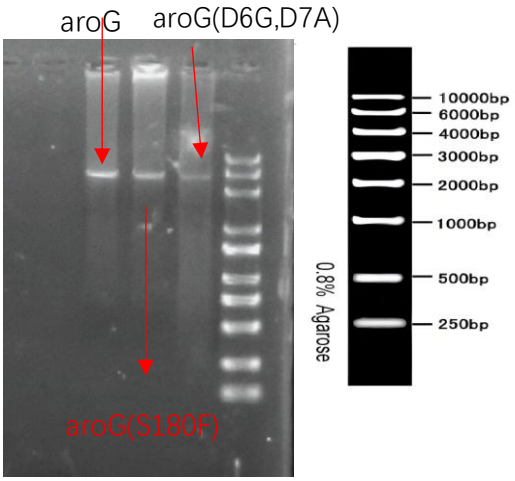
PCR system(50ul)	
2 Mix	25ul
aroGS211F-F	2ul
aroGS211F-R	2ul
Template	50ng
DDW	20ul

PCR system(50ul)	
2 Mix	25ul
aroGS180F-F	2ul
aroGS180F-R	2ul
Template	50ng
DDW	20ul

The PCR products were analyzed by agarose gel electrophoresis, revealing the correct target fragment length of 1572 bp. We obtained the correct target fragment and proceeded with gel extraction.

2. Product Purification

The PCR products were analyzed by agarose gel electrophoresis, showing band sizes of 5395 bp with correct positions. The products were purified and recovered. The results are as follows:



3. DpnI Digestion

The product will undergo digestion in the following digestive system:

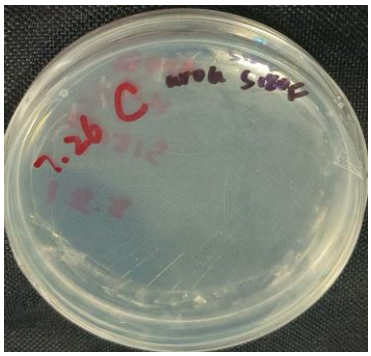
Digestion system	
DpnI	0.2μL
cutsmart	1μL
aroG（S180F）	1.7μL
ddH2O	7.1μL

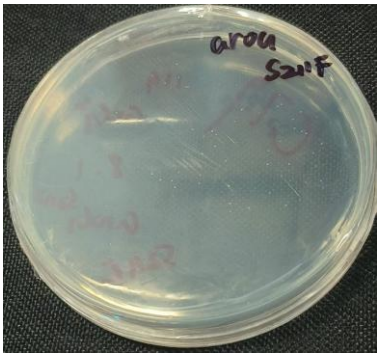
Digestion system	
DpnI	0.2μL
cutsmart	1μL
aroG（D6G-D7A）	2.8μL
ddH2O	6μL

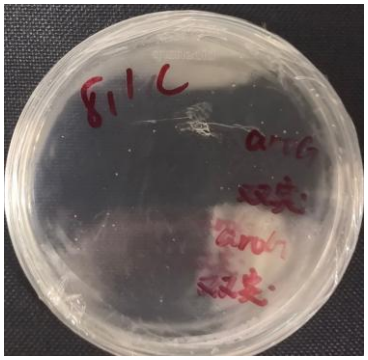
Digestion system	
DpnI	0.2μL
cutsmart	1μL
aroG（S211F）	3.3μL
ddH2O	5.5μL

4. Chemical Conversion

Using chemical transformation, the digested PCR products were transferred into DH5α competent cells and spread onto LB plates containing chloramphenicol resistance. The plates were incubated overnight at 37°C. The expected resistant single colonies grew on the plates, exhibiting good bacterial growth.







5. Plasmid extraction

The sequencing results for pSB1c-p23119-aroGD6G-D7A, pSB1c-p23119-aroGS180F, and pSB1c-p23119-aroGS211F are correct.

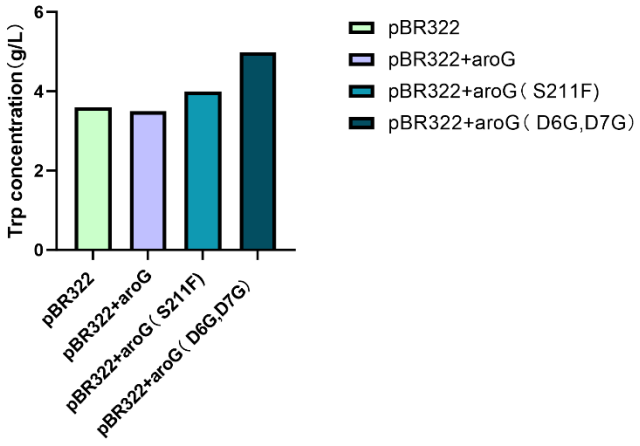
（B）Fermentation

1.Double fermentation:

Transform pSB1c-p23119-aroGD6G-D7A, pSB1c-p23119-aroGS180F, and pSB1c-p23119-aroGS211F into BWΔtrpRΔtnaABΔpheA competent cells. Plan to conduct dual-transformation fermentation yield tests.

（C）Liquid-phase testing

Tests were conducted at 0h, 12h, 24h, 30h, 36h, and 48h. Results showed that under identical conditions, the aroG double mutant showed the highest tryptophan production, approximately 5g/L, which was 1.25 times higher than that of the control group. Moreover, the results indicated that the tryptophan production of the aroG mutant was higher than that of PBR322 and aroG.



（D）The second round selection for bacterial strains with high Atcomt enzyme activity

1.Perform error-prone PCR

PCR system (Mg^{2+}：Mn^{2+}=10：1）	
2×Mix Green Master	25μl
Mg^{2+}(10mM)	1μl
Mn^{2+}(1mM)	1μl
AtCOMT-R	2μl
AtCOMT-F	2μl
Templete	50ng
DDW	to 50μl

2. Product purification

The products of error-prone PCR were purified

3.connection

The plasmid pYB1a-AtCOMT* was constructed by connecting with golden gate 10 tubes. The connection system is as follows:

BsaI	0.5μL
rCutSmart Buffer	1μL
T4 DNA Ligase	0.2μL
10×Ligase Buffer	1μL

ddH2O	to 10μL
PYB1a-ccdB	150ng
AtCOMT***	150ng

37℃	30 min
37℃	3 min
16℃	3 min
16℃	20 min
37℃	20 min
55℃	15 min
80℃	15min

After the connection, unlike the first round of screening, we chose to convert it into DH5α and then coated it:

4.Mixing plasmids were obtained by plate washing

Take 1ml LB solution, wash the colonies on 10 plates into a 100ml conical flask, and obtain mixed plasmid after 12h of culture. Transform the obtained plasmid into 13 tubes of DH5α again, and extract mixed plasmid again.

5.Transformation and transition

The hybrid plasmid was transformed into the cysteine-deficient strain BWΔCysE-PLB1s-Cys3-Cys4. After 12-hour plate incubation at 37℃°C, bacterial colonies from 13 plates were washed and transferred to a 100ml conical flask. 50μL of the mixture was then transferred to M9 medium supplemented with Cysteine for 30℃ hours of transition. The subsequent 30-40 hour transition period utilized the following transformation parameters:

5ml Filtering system	
M9	1mL
AMP,Str	Each 5μL
20% glycerinum	0.5ml

MgSO4	10μL
CaCl2	0.5μL
Ara	50μL
Cys	0.2mM
H2O	3.5ml

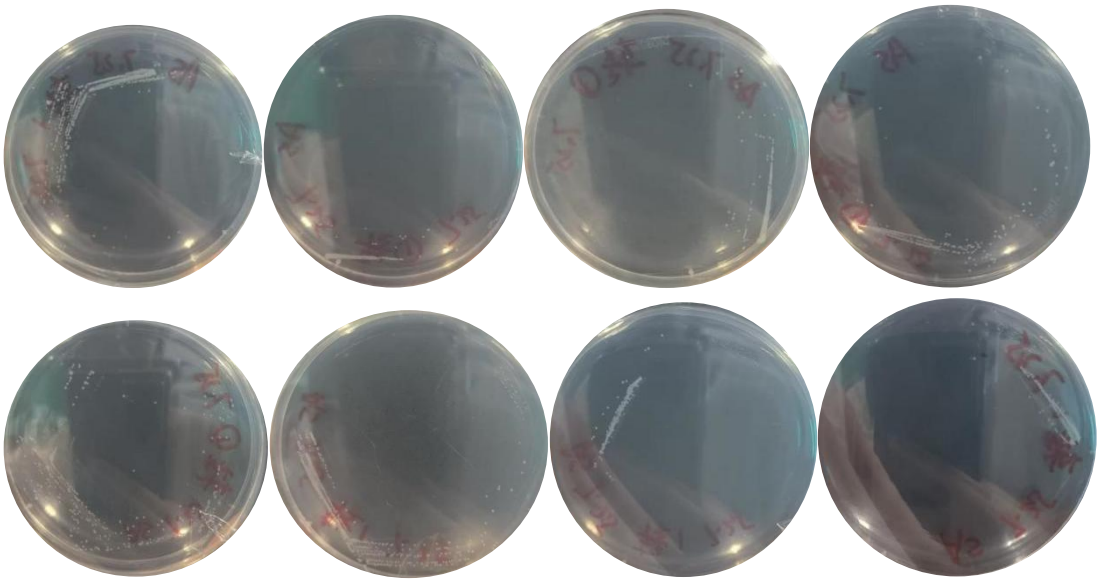
6.selection

250μL of bacterial fluid was taken from the transition system and transferred to the screening system for screening. The screening system is as follows：

5ml Screening system	
M9	1mL
20% glycerinum	0.5ml
MgSO4	10 μL
CaCl2	0.5 μL
Ara	50 μL
Bacteria liquid	250μL
H2O	3.5ml
SAM	100μL（2mM）
NAS	100μL（1mM）

7.Obtain a library of single-cloned mutants

Cultivate the bacteria in the screening system for 16h. After the bacterial liquid becomes slightly turbid, 250μL of the bacterial liquid is drawn into the screening system. Repeat the process until the bacterial liquid becomes slightly turbid again. Then make a plate streak and pick a single colony for inoculation.



(E) Tryptophan synthesis levels of the mutant bacteria in the 15th round of fermentation and the 5.1/10.6/10.7 mutant bacteria in the last round of fermentation:

Control group: BWΔtrpRΔtnaAB-pYB1a-P23119-tnaE^{S40F}DCBA

Experimental group: 10 mutant strains from the 15th round, and 5.1/10.6/10.7 mutant strains

1. Plate streaking method was used to isolate monoclonal clones:

The 15th round mutant bacteria were streaked on plates (LB plates with Amp, Smr and Kana resistance), and the monoclones were isolated.

2. Single clone inoculation:

Randomly select 10 monoclones from the streaked plates of the 15th round of mutant bacteria and inoculate them into liquid LB medium. The BWΔtrpRΔtnaAB-pYB1a-P23119-tnaE^{S40F}DCBA strain and the 5.1/10.6/10.7 mutant bacteria were inoculated from the culture tubes and incubated at 37 ° C for 12 hours.

3. Coating plate:

The bacterial solution was dipped in three times from liquid LB medium with an inoculation ring and spread onto the corresponding resistant LB plates of each strain until paste-plate and incubated for 12h at 37℃.

4. Inoculation into seed medium:

The colonies on the plates were scraped off with an inoculation ring, inserted into the seed medium, and incubated for 18h in a shaker at 37℃ until an OD600 of 1-1.2 was achieved.

5. Inoculation into fermentation medium:

1.2OD of the bacterial solution was taken into a 10ml EP tube, the centrifuge was precooled at 4℃, and the centrifugation program was set to 4200 RPM for 10min.

The fermentation medium was prepared.

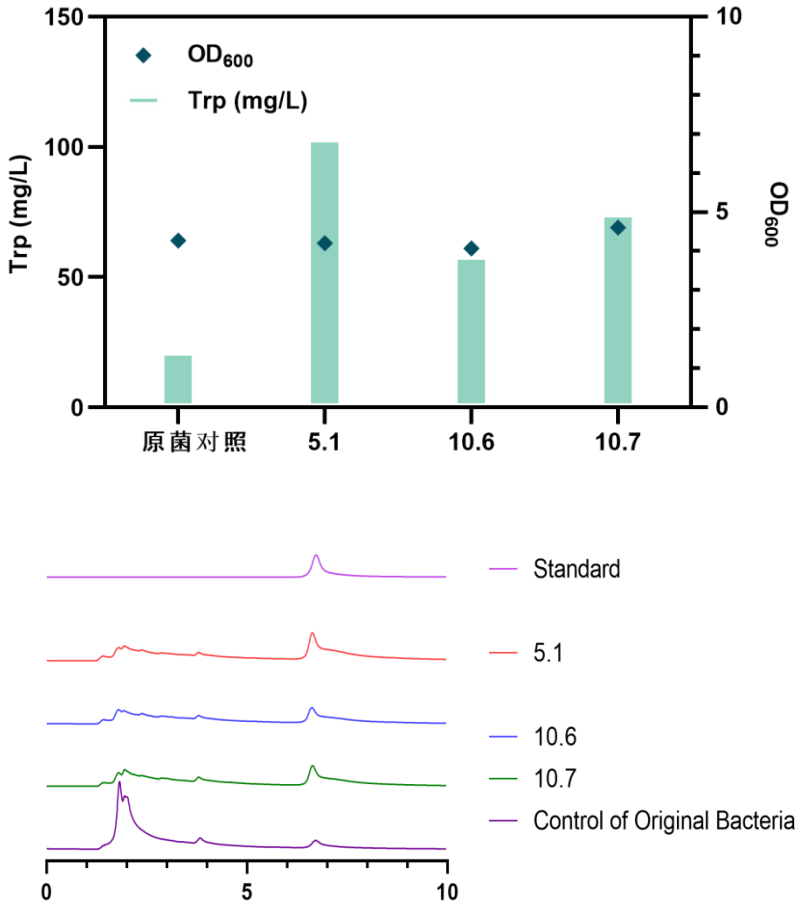
After centrifugation, the supernatant in the 10ml EP tube was discarded, 300ul of the prepared fermentation medium was sucked into the EP tube after centrifugation, the colonies were blown and mixed, and all the liquid in the EP tube was poured back into the medium and incubated in a shaker at 37 ℃.

6. Spectrophotometer measurement of OD600 value, liquid phase sample preparation:

500μL of bacterial solution was taken from the conical flask where each strain was located, and 200μL of bacterial solution was taken to measure the OD600 value with a spectrophotometer. At 28h, the growth of all strains reached the plateau according to OD value.

The remaining 300μL of bacterial solution was used for sample preparation, and after centrifugation at 10000g for 10min, the supernatant was aspirated with a syringe, filtered through the membrane, and poured into a liquid phase vial.

7. High performance liquid chromatography (HPLC) detection of tryptophan production:



None of the 10 mutants selected in the fifteenth round produced tryptophan.

Week4: 8.22-8.28:

(A) AtCOMT enzyme activity in E. coli strains selected by HPLC

1.induction

The filtered Bw25113△CysE/pLB1s-Cys34/pYB1a-AtCOMT* were respectively inoculated into 5ml LB liquid culture medium containing 0.1% ampicillin and 0.1% streptomycin, and cultured at 30℃ for 16h. The induction system was as follows:

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

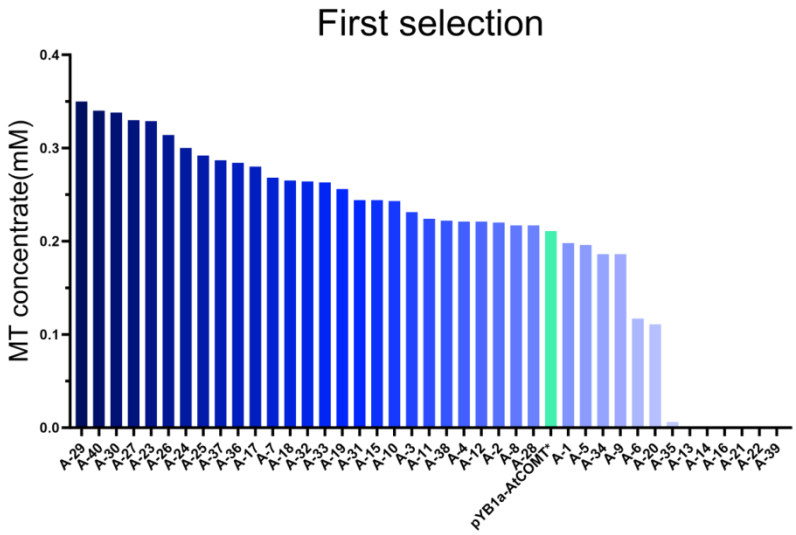
2.Whole cell catalysis

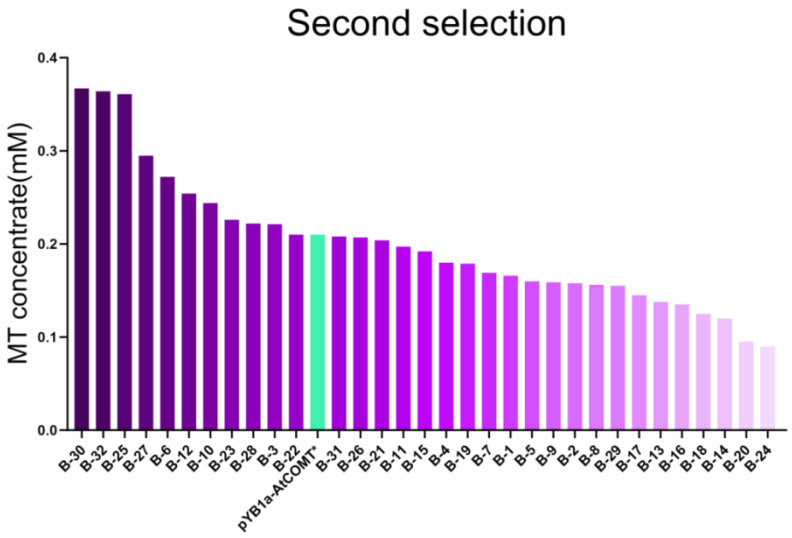
After induction, the bacterial suspension of 6OD was taken to a 2ml EP tube. After centrifugation, the supernatant was discarded and the whole cell catalysis was carried out. The whole cell catalysis system was as follows:

SAM	2mM
1mM NAS tris-HCl	200μl

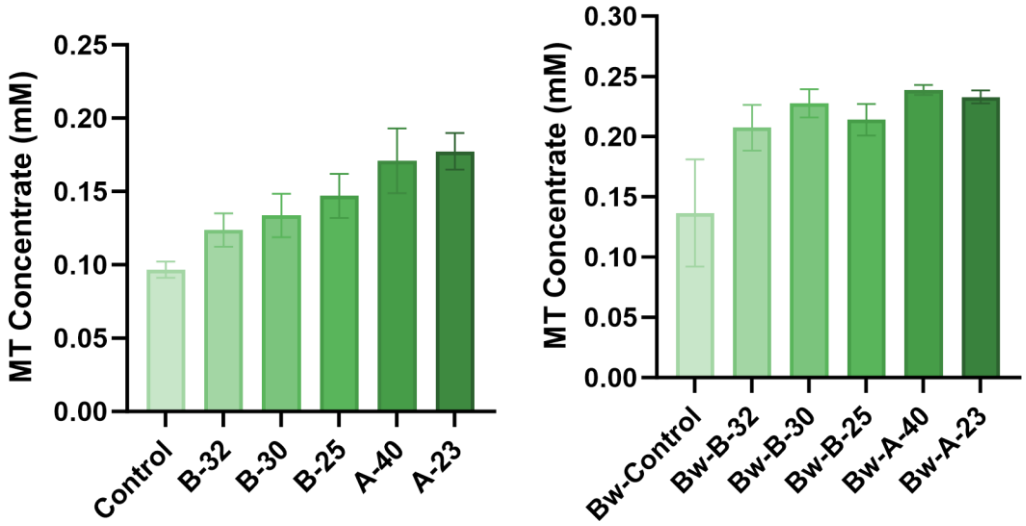
3.HPLC check

Samples prepared after whole-cell catalysis were used for liquid-phase detection. Subsequently, these strains underwent sequencing analysis to confirm their mutation status and potential functional characteristics. The HPLC results from two rounds of library screening are as follows:





As shown in the figure, 27 out of the 40 strains selected for detection in the first screening cycle exceeded the original AtCOMT*** template, while 11 out of the 32 strains selected in the second screening cycle surpassed the same benchmark. To prevent Cys auxotrophic strains from affecting enzyme activity, we treated the top-performing five strains from both screenings with the pLB1s-Cys34 plasmid elimination process. Subsequent retests were conducted on Bw25113 and Bw25113ΔCysE strains, yielding the following results:



Bw25113ΔCysE/pLB1s-Cys3-Cys4/pYB1a-AtCOMT* Bw25113/pYB1a-AtCOMT*

The results showed that the selected enzyme with the highest activity AtCOMT* improved by about 84% compared with the existing template with the highest activity. Molecular docking simulation will be carried out in the future.

(B) Test the tryptophan synthesis level of the 5.1/10.6/10.7 mutant strains through repeated fermentation in a 15ml system:

Control group: BWΔtrpRΔtnaAB-pYB1a-P23119-tnaE^{S40F}DCBA

Experimental group: 5.1/10.6/10.7 mutant strain

Set up three groups of parallel experiments

1. Plate streaking method was used to isolate monoclonal clones:

The 15th round mutant bacteria were streaked on plates (LB plates with Amp, Smr and Kana resistance), and the monoclones were isolated.

2. Selective monoclonal inoculation:

Randomly select 10 monoclones from the streaked plates of the 15th round of mutant bacteria and inoculate them into liquid LB medium. The BWΔtrpRΔtnaAB-pYB1a-P23119-tnaE^{S40F}DCBA strain and the 5.1/10.6/10.7 mutant bacteria were inoculated from the culture tubes and incubated at 37℃ for 12 hours.

3. Coating plate:

The bacterial solution was dipped in three times from liquid LB medium with an inoculation ring and spread onto the corresponding resistant LB plates of each strain until paste-plate and incubated for 12h at 37℃

4. Inoculation into seed medium:

The seed culture medium was used in 250 ml conical flasks, and the system ratio was as follows:

Seed culture medium system (15ml)	
Seed culture medium	13.5ml
Seed glucose	1.5ml
Antibiotics corresponding to strains	15ul

The colonies on the plates were scraped off with an inoculation ring, plugged into seed medium, and incubated in a shaker at 37 ℃ for 18h until an OD of 5-6.

5. Inoculation into fermentation medium:

6 OD of the bacterial solution was taken into a 10ml EP tube, the centrifuge was precooled at 4℃, and the centrifugation program was set to 4200 RPM for 10min.

A 250 ml conical flask was used as a container in the ultra-clean table to configure the fermentation medium. The system ratio was as follows:

Fermentation culture medium system (15ml)	
Fermentation medium	12ml
Seed culture medium	1.5ml
Fermented glucose	1.5ml
Antibiotics corresponding to strains	15ul

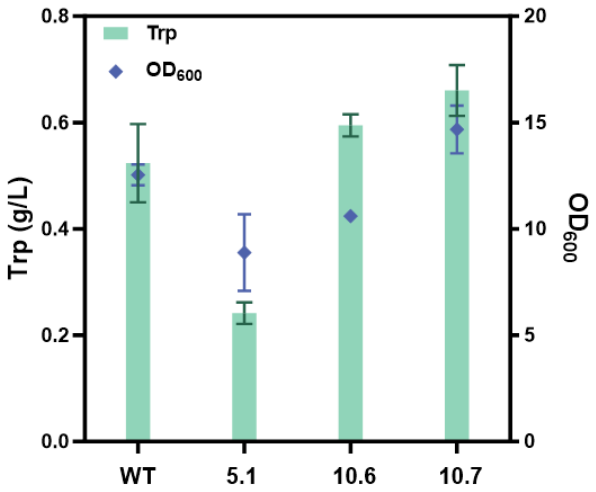
After centrifugation, the supernatant in the 10-ml EP tube was discarded, 300ul of the prepared fermentation medium was sucked into the EP tube after centrifugation, the colonies were blown and mixed, and all the liquid in the EP tube was poured back into the medium and incubated in a shaker at 37 ℃.

6. OD600 value measured by spectrophotometer and liquid phase sample preparation:

500μL of bacterial solution was taken from the conical flask where each strain was located, and 200μL of bacterial solution was taken to measure the OD value with a spectrophotometer. At 28h, the growth of all strains reached the plateau according to OD value.

The remaining 300μL of bacterial solution was used for sample preparation, and after centrifugation at 10000g for 10min, the supernatant was aspirated with a syringe, filtered through the membrane, and poured into a liquid phase vial.

7. High performance liquid chromatography (HPLC) detection of tryptophan production:



Compared with the WT strain, the 10.7 mutant strain increased Trp production by 25% in shake flask fermentation.

(C) Rounds 1-10 whole-genome mutation fermentation tests:

Control group: BWΔtrpRΔtnaAB-pYB1a-P23119-tnaE^{S40F}DCBA

Experimental group: 4 strains from round 1 to round 10 were tested for whole-genome mutation (pre-experiment)

1. Plate streaking method was used to isolate monoclonal clones:

The mutant strains from rounds 1 to 10 were plate streeted (LB plate containing Amp, Smr, Kana resistance), and monoclonal clones were isolated.

2. Selective monoclonal inoculation:

Randomly select 10 monoclones on the streaked plates of mutant bacteria from round 1 to Round 10 and inoculate them into liquid LB medium. Inoculate the BWΔtrpRΔtnaAB-pYB1a-P23119-tnaE^{S40F}DCBA strain from the culture tube and incubate at 37℃for 12 hours.

3. Inoculate into seed culture medium:

Enrich the bacterial cells in LB, inoculate them into the seed culture medium, use 50 ml EP tubes as containers, and incubate in a 37℃ shaker for 18 hours until the OD600 is 1-1.2.

4. Inoculate into the fermentation medium

1.2OD of the bacterial solution was taken into a 10ml EP tube, the centrifuge was precooled at 4℃, and the centrifugation program was set to 4200 RPM for 10min.

The fermentation medium was configured using 50 ml EP tubes as containers in an ultra-clean table. The supernatant of 10ml EP tube after centrifugation was discarded, 300ul of the prepared fermentation medium was sucked into the EP tube after centrifugation, the colonies were blown and mixed, and all the liquid in the EP tube was poured back into the medium and incubated in a shaker at 37 ℃.

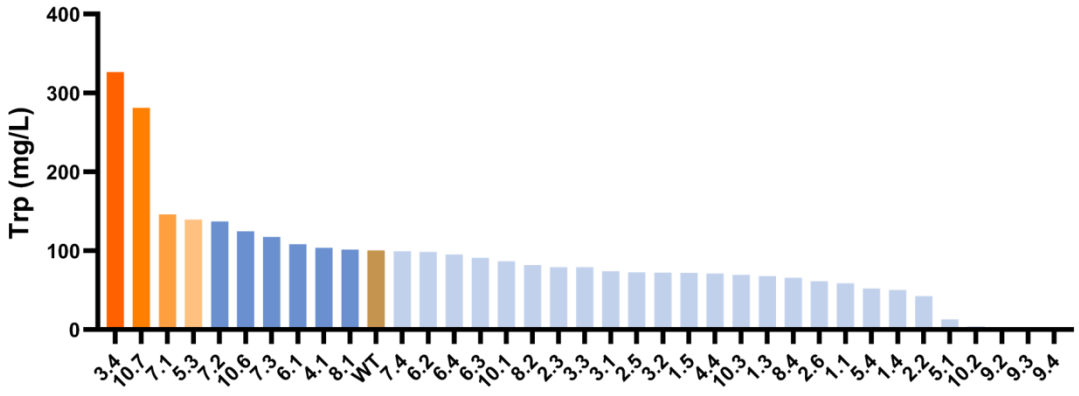
5. Spectrophotometer OD value, liquid phase sample preparation:

500μL of bacterial solution was taken from the conical flask where each strain was located, and 200μL of bacterial solution was taken to measure the OD600 value with a spectrophotometer. At 28h, the growth of all strains reached the plateau according to OD600 value.

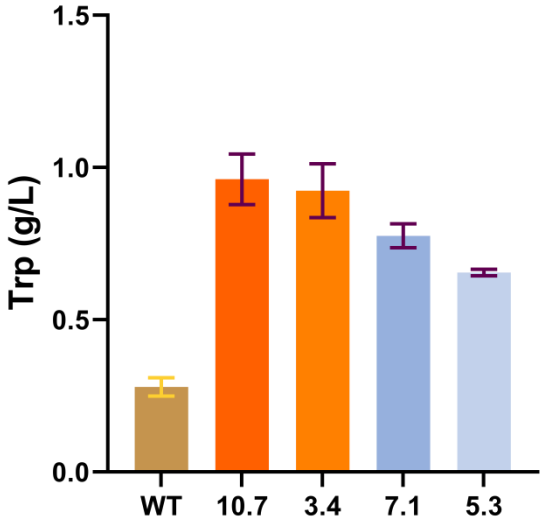
The remaining 300μL of bacterial solution was used for sample preparation, and after centrifugation at 10000g for 10min, the supernatant was aspirated with a syringe, filtered through the membrane, and poured into a liquid phase vial.

6. High performance liquid chromatography (HPLC) detection of tryptophan production:

Mutant strains 3.4 and 10.7 were found to increase tryptophan production by about 300% compared with the wild type, and the experiment will be repeated.



Subsequently, the highest yield strains 10.7, 3.4, 7.1, 5.3, wild type control group were re-fermentation test, set up three groups of parallel experiments, the results are as follows:



The mutants 10.7 and 3.4 were confirmed to have significantly increased Trp production, up to three times that of the wild type, and will be re-analyzed by the whole genome.

(D) Overexpression of yddG/glnA/prs to test the level of tryptophan synthesis:

Control group: BWΔtrpRΔtnaAB/PBR322-TRP

Experimental group: Using BWΔtrpRΔtnaAB/PBR322-TRP as the chassis strain, pSB1c-P23119-yddG/pLB1s-P23107-glnA-prs were combined and introduced

Three groups of parallel experiments were set up.

1. Chemical transformation

Chemical transformation into BWΔtrpRΔtnaAB, the plasmids were:

① pLB1s-P23107-glnA-prs + pBR322-trpEDCBA^{fbr}-aroG^{fbr}-serA^{fbr}
② pSB1c-P23119-yddG + pBR322-trpEDCBA^{fbr}-aroG^{fbr}-serA^{fbr}
③ pLB1s-P23107-glnA-prs + pSB1c-P23119-yddG + pBR322-trpEDCBA^{fbr}-aroG^{fbr}-serA^{fbr}
④ pBR322-trpEDCBA^{fbr}-aroG^{fbr}-serA^{fbr}

The plates were coated on LB plates containing the corresponding resistance and incubated at 37°C for 12h.

2. Single clone inoculation:

Monoclonal clones were selected and inoculated into liquid LB medium. The BWΔtrpRΔtnaAB-pYB1a-P23119-tnaE^{S40F}DCBA strain was inoculated from the storage tube and incubated at 37°C

for 12 hours.

3. Inoculation into seed medium:

Enrich the bacterial cells in LB, inoculate them into the seed culture medium, and use a 250 ml conical flask to culture in a 37℃ shaker for 18 hours until the OD600 is 5-6

4. Inoculate into the fermentation medium

Take 6OD of the bacterial liquid into a 10ml EP tube, pre-cool it in a centrifuge at 4 ℃, and set the centrifugation program to 4200 RPM for 10 minutes.

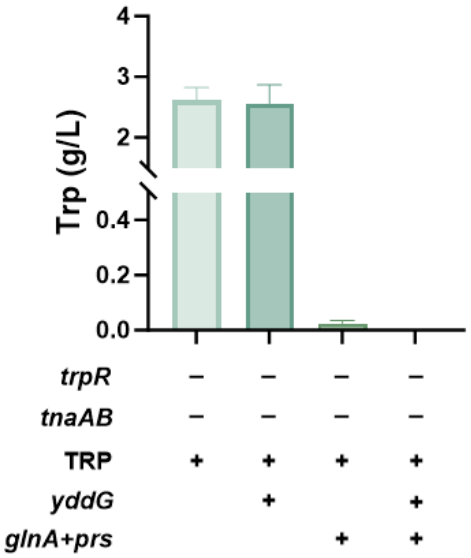
Use a 250 ml conical flask as the container in the laminar flow hood to prepare the fermentation medium. Discard the supernatant in 10ml of the EP tube after centrifugation. Aspirate 300 μl of the prepared fermentation medium into the EP tube after centrifugation, and mix the colonies by pipetting. Pour all the liquid in the EP tube back into the medium and place it in a 37℃ shaker for culture.

5. Spectrophotometer OD value, liquid phase sample preparation:

500μL of bacterial solution was taken from the conical flask where each strain was located, and 200μL of bacterial solution was taken to measure the OD value with a spectrophotometer. At 28h, the growth of all strains reached the plateau according to OD value.

The remaining 300μL of bacterial solution was used for sample preparation, and after centrifugation at 10000g for 10min, the supernatant was aspirated with a syringe, filtered through the membrane, and poured into a liquid phase vial.

6. High performance liquid chromatography (HPLC) detection of tryptophan production:



Result analysis

Overexpression of yddG did not increase the production of tryptophan, suggesting that the expression of yddG was sufficient.

Overexpression of glnA/prs led to a significant decrease in tryptophan production, suggesting a change in carbon flux.