WEEK 17

7.21 Peng Jiang

Engineering bacteria

BL21/*tyrB*, *tyrA* knockout preparation.

Amplification of *tyrB* targeting fragments.

Because the upstream and downstream fragments amplified by the previous set of primers were used for overlap ligation, multiple specific bands appeared many times, so the primers were redesigned.

over-F-tyrB-F--3	ATAAAGCCGGAGAGCCTGTTGGTTATG
over-F-tyrB-R--3	CTTTACCTGTTGAGCTAGATGCAC

Use this primer to re-amplify the fragment.

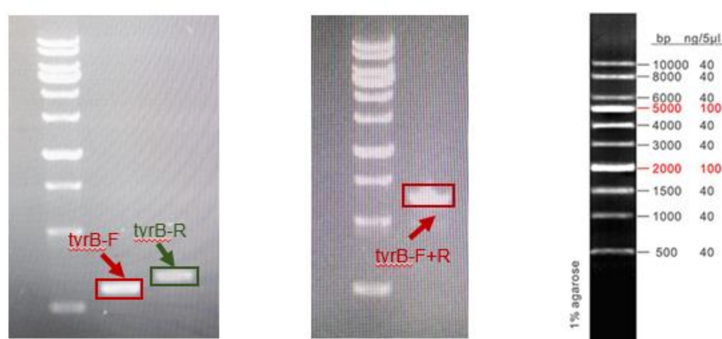

Figure 1 tyrB-F, tyrB-F and tyrB-F+R detection gel map

TyrB knockout.

The first experiment process:

pTarget-tyrB: 1 μL/tyrB-F+R: 6 μL

Add the electro-transfer competencies and let stand for 20 min.

Move into the electro-rotor cup, program ec1, shock time 4.8.

Quickly add LB medium for 1 min, incubate at 37°C for 1 h, centrifuge and plate, overnight.

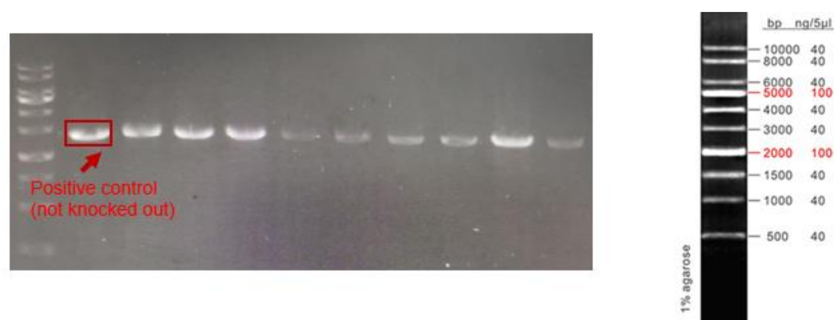

Figure 2 Gel image of colony PCR detection

Then perform electrophoresis verification on the target fragments:

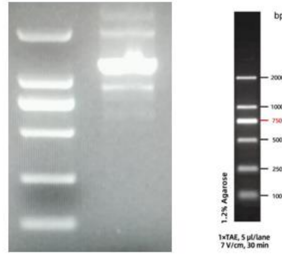

Figure 3 The gel image of the target fragment electrophoresis verification gel

It was found that there were too many miscellaneous bands, so after the gel was recovered, it was used as a template for amplification. As a result, there were still a large number of miscellaneous banuLds. Recycle all products with a concentration of 35 ng/μL.

The second experiment process:

pTarget-tyrB: 1 μL tyrB-F+R: 9 μL

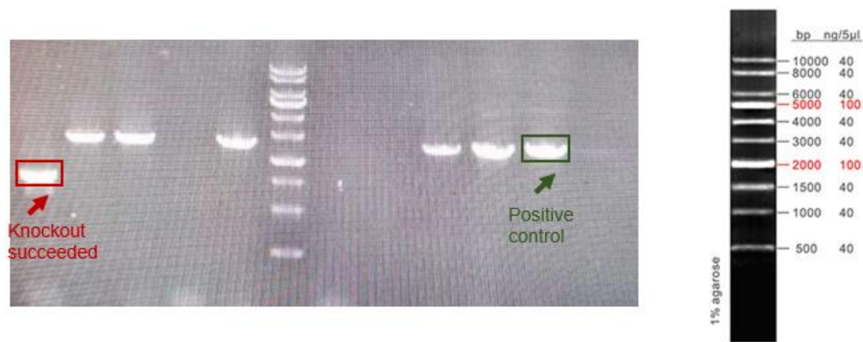

Figure 4 *tyr-B* knockout detection gel

pTarget-tyrR construction

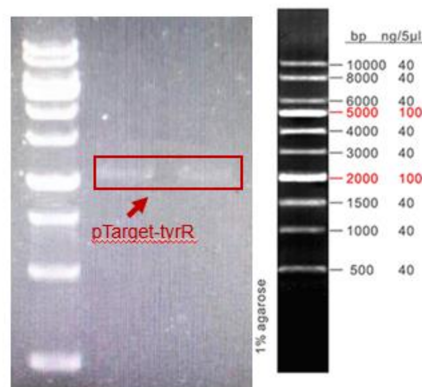

Figure 5 *pTarget-tyrR* construction detection gel map

The PCR product was transferred to T1 and sequenced, showing no signal. Redesign the primers as follows:

pTarget-tyrR-F-2	ctagtCTCGATCTACTCGTGCTAAGgttttagagctagaaatagc
pTarget-tyrR-R-2	aaaacCTTAGCACGAGTAGATCGAGactagtattatacctaggac

7.22 Shuhan Liu

Changes in the calculation method of induction strength

The calculation method of induction strength has been changed to make the calculation of induction strength more accurate.

Background level expression intensity (I0) = (F background expression-FDH5α not transfected into plasmid)/OD background expression.

4HB induction intensity (IB) = (F4HB induction-FDH5α without plasmid transfer)/OD4HB induction

HMA induction intensity (IA) = (FHMA induction-FDH5α without plasmid transfer)/ODHMA induction

Analogue induction intensity (I analogue) = (F analogue induction-FDH5α not transfected)/OD analogue induction

Note: F is the fluorescence reading of the microplate reader.

7.23 Chao Chen

Re-screening

Re-screening the bacteria with site-directed saturation mutation of D12, and re-processing the data of F1-F10, M (site-directed mutation)-F1, and M-F2.

The re-screening of D12 site-specific saturation mutations was completed. At present, a total of 11 bacteria have entered the re-screening verification.

Use the new calculation method to recalculate the re-screening data:

A total of 647 strains (of which 8 strains appear twice in the figure), their induction is shown in the figure.

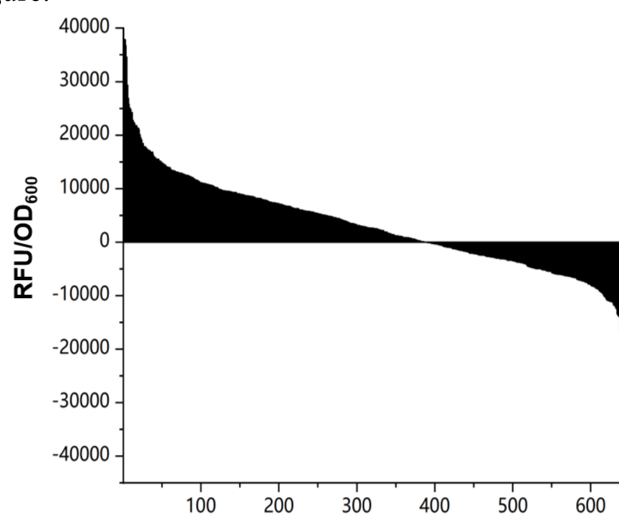

Figure 6 Re-screening data chart produced by 647 strains

According to the new calculation method, select the top 49 strains (including two duplicate strains) with a higher degree of induction than the DH5α control bacteria IB.

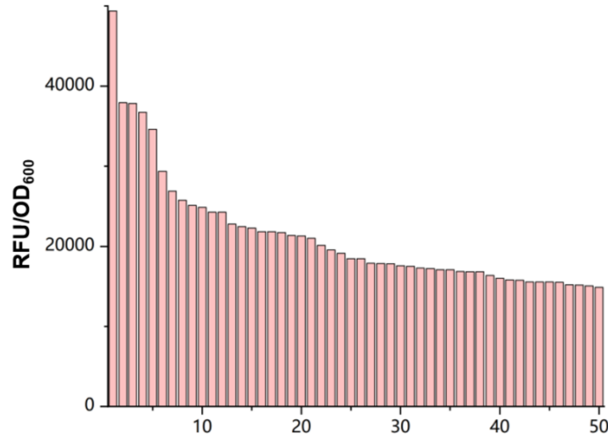

Figure 7 Data map of 49 strains

The second round of re-screening is calculated using the new calculation method, and 47 bacteria that respond well to HMA can be obtained, as shown in the figure below.

F1-D9	F5-H1	F9-A11	F4-G4	F2-G9	F3-G6	F9-A3
F9-A7	F4-A11	F9-C6	F4-C4	F3-A7	F1-H6	F1-B4
F3-H3	F1-C3	F4-D5	F3-E5	F3-H4	F2-B9	F1-E7
F9-C4	F2-A5	F5-C9	F6-H4	F9-A12	F9-A10	F3-B7
F2-B4	F4-G11	F4-C7	F2-C12	F9-A6	F4-A9	F3-H7
F3-F3	F4-C12	F1-D4	F5-B10	F1-D5	F9-A9	F9-C12
F4-A12	F5-G1	F3-C4	F7-D2	F3-G3	F9-A8	F7-D12

Figure 8 A table of 47 bacteria that respond well to HMA

Note: F1-D9 and F5-H1 are the same strain; F1-E7 and F5-G1 are the same strain, which is a repeated experiment.

7.24 Fengqianrui Chen

Re-screening verification

Re-screening and verifying the bacteria with better expression in the D12 point mutation re-screening, re-processing the data of T1-T11, M-T1, and M-T2; calculating by a new calculation method, 47 strains with a good degree of induction were selected for re-screening verification.

Perform re-screening verification on the strains selected in the D12 site-specific saturation mutation re-screening. The following is a summary of the re-screening verification data of the 11 strains selected in the re-screening.

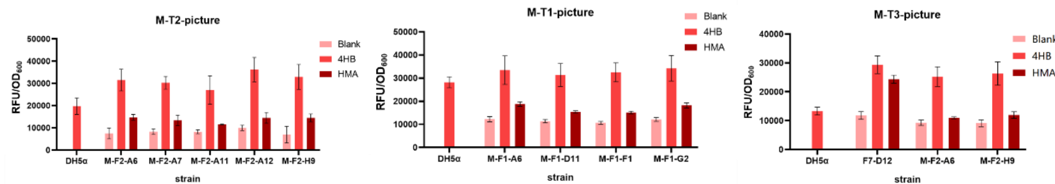

Figure 9 Re-screening verification data of 11 strains

The background level expression of all apex mutant bacteria was higher, and the HMA response was lower than that of 4HB.

There is no better strain than D12.

Subsequently, we conducted a secondary verification of whether the strains screened out by the re-screening responded to HMA, and at the same time excluded false positives caused by the off-target PobR protein.

Perform re-screening verification in accordance with the original re-screening verification system, using a new calculation method to calculate the induction value, and sorting in descending order according to IA/I0. Part of the results are shown below. We found that F3-G3, F3-C4, and F3-E5 had a higher response to HMA, so we selected them for further verification.

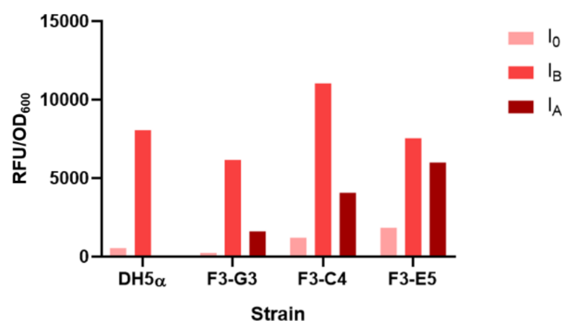

Figure 10 Re-screening verification data map

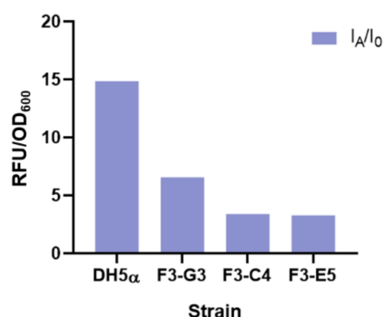

Figure 11 Re-screening verification data map (II)

According to the new re-screening verification system for re-screening verification, using a new calculation method to calculate the induction value, we found that 49 strains have a low response to HMA. The results are shown below.

1	F1-B4	8	F2-A5	15	F3-C4	22	F3-H7	29	F4-D5	36	F6-H4	43	F9-A3
2	F1-C3	9	F2-B4	16	F3-E5	23	F4-A11	30	F4-G11	37	F7-D12	44	F9-A6
3	F1-D4	10	F2-B9	17	F3-F3	24	F4-A12	31	F4-G7	38	F7-D2	45	F9-A7
4	F1-D5	11	F2-G2	18	F3-G3	25	F4-A9	32	F5-B10	39	F9-C6	46	F9-A8
5	F1-D9	12	F2-G9	19	F3-G6	26	F4-C12	33	F5-C9	40	F9-A10	47	F9-A9
6	F1-E7	13	F3-A7	20	F3-H3	27	F4-C4	34	F5-G1	41	F9-A11	48	F9-C12
7	F1-H6	14	F3-B7	21	F3-H4	28	F4-C7	35	F5-H1	42	F9-A12	49	F9-C4

Figure 12 Table of 47 bacteria with low response to HMA

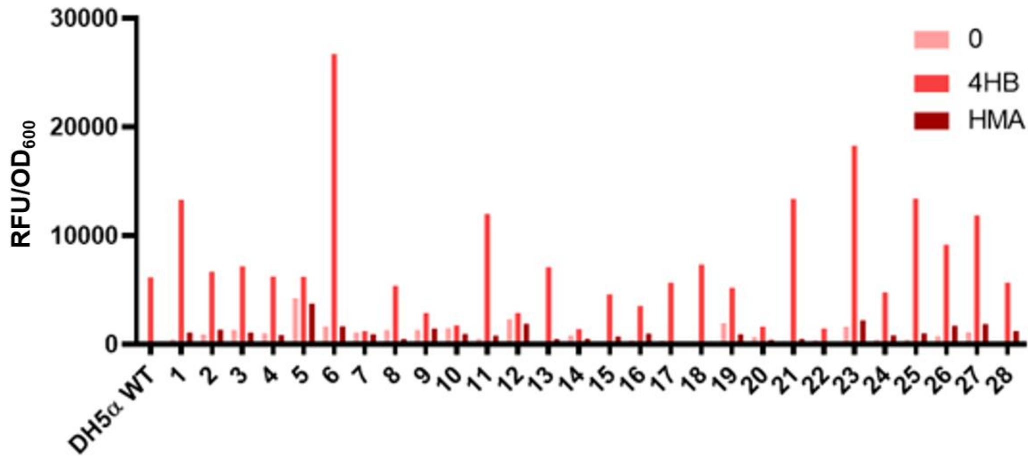

Figure 13 Re-screening verification data map

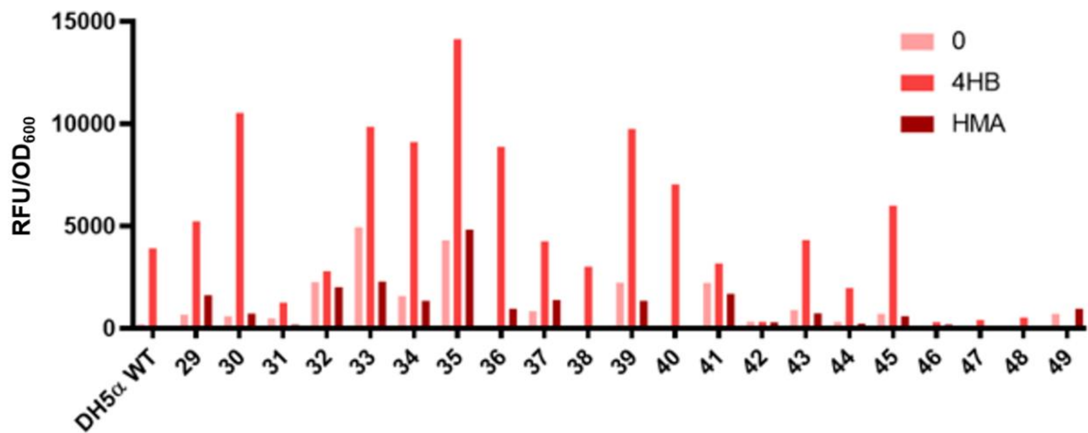

Figure 14 Re-screening verification data map (II)

The 49 sets of data were arranged in descending order of IA/I0, and 6 strains of bacteria that responded well to HMA were obtained, displayed as shown in the figure, and the next step of re-screening was performed for verification.

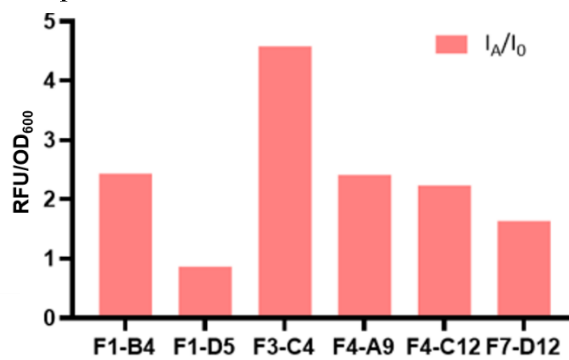

Figure 15 Data diagram of 6 strains of bacteria that responded well to HMA

We tested the 6 strains that responded well to HMA for the next step of re-screening and verification.

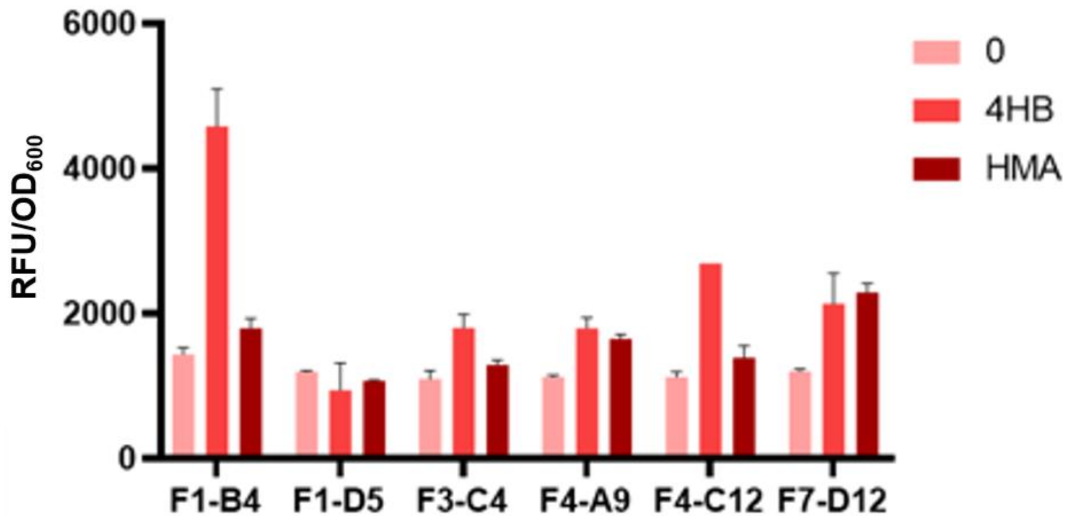

Figure 16 The next step of re-screening verification data of 6 strains that have a better response to HMA

We found that DH5α plasmid-free bacteria did not grow, and the OD of 4HB-induced strains was generally lower than that of non-induced and HMA-induced strains.
Our preliminary analysis is that the 4HB concentration is too high, which is not conducive to bacterial growth.
It is planned to reduce the 4HB concentration and re-screening verification.

7.25 Linxi Jiang

HMA gradient verification
Re-validate the HMA gradient for strains with a higher degree of induction (F3-G3, F3-C4, F3-E5)
Perform HMA gradient verification on the bacteria with better induction degree (F3-G3, F3-C4, F3-E5), and further determine the HMA concentration range

Control group:
200 μL LB+1% DH5α non-transformed plasmid bacterial solution
200 μL LB+1‰ 50 mg/mL ampicillin+1% DH5αPobR^{WT} bacterial solution
196 μL LB+1‰ 50 mg/mL ampicillin+ 4 μL 50 g/L 4HB+1% DH5αPobR^{WT} bacterial solution

Experimental group (200 μL system):
LB+HMA+1‰ 50 mg/mL ampicillin+1% bacterial solution (HMA concentration is 0, 0.01, 0.05, 0.08, 0.1, 0.5, 0.8, 1g/L)

Note: The concentration of HMA mother liquor used is 6 g/L and 50 g/L.

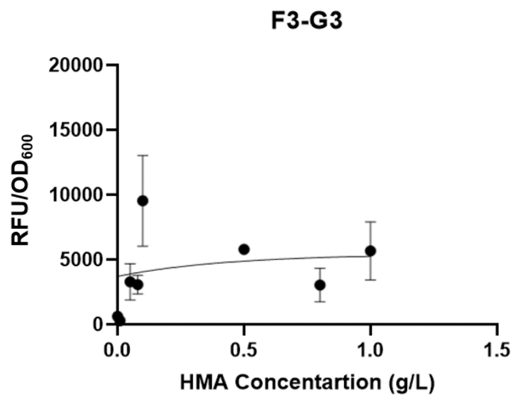

Figure 17 HMA gradient verification results of F3-G3

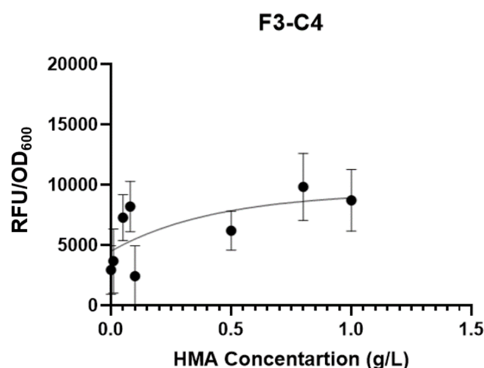

Figure 18 HMA gradient verification results of F3-C4

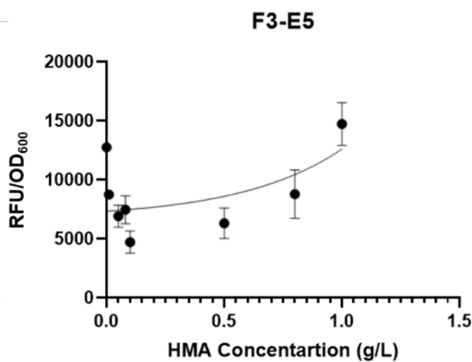

Figure 19 HMA gradient verification results of F3-E5

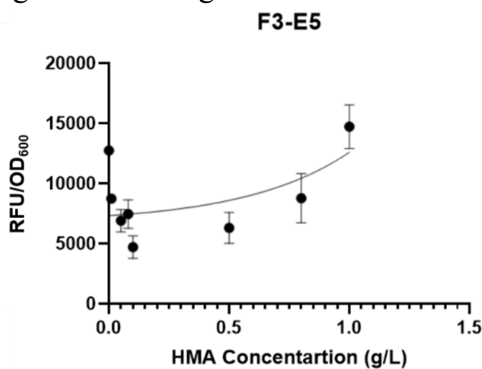

Figure 20 HMA gradient repeated verification results of F3-E5

A new round of HMA gradient verification was performed on F3-E5, which is repeatable, but the error is large, so it is discarded.

In the repeated experiments for verification of re-screening, the induction value of the same strain was unstable, so it was decided to increase the inducer concentration to 1 g/L to ensure the stability of the induction intensity.
And because the experimental strains are all DH5α type, DH5α plasmid-free bacteria were selected as the blank control.

7.26 Bowen Zhang

HMA analog response verification

Response verification of old HMA analogues:
Use HMA analogs to induce D12, compare the induced fluorescence value with the fluorescence value induced by LB and HMA to test its specificity.

Control group:
200 μL LB+1‰ 50 mg/mL ampicillin
200 μL LB+1‰ 50 mg/mL ampicillin+1% DH5αPobR^{WT} bacterial solution
196.6 μL LB+1‰ 50 mg/mL ampicillin+ 3.4 μL 6 g/L 4HB+1% DH5αPobR^{WT} bacterial solution (final concentration is 0.1 g/L)
196.6 μL LB+1‰ 50 mg/mL ampicillin+ 3.4 μL 6 g/L HMA+1% DH5αPobR^{WT} bacterial solution (final concentration is 0.1 g/L)

Experimental group (200 μL system): LB+ analogs+1‰ 50 mg/mL ampicillin+1% bacterial solution (analogs include MA, HPP, Phe, Tyr, PPA, phenylethanol, phenylacetaldehyde, Trp)

The concentration of the analog mother liquor used is 5 g/L.

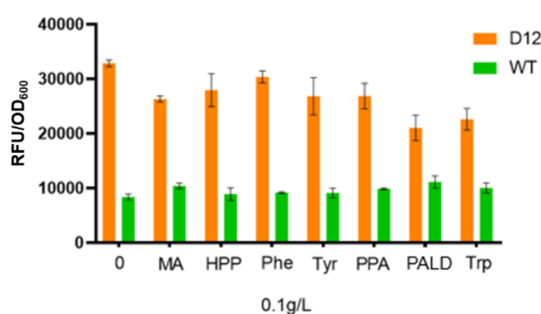

Figure 21 Induction data graph of D12 and DH5αPobR^{WT} by different inducers
The results show that the background expression of F7-D12 is too high

New HMA analog response verification:
Use HMA analogs to induce DH5αPobR^{WT} and F7-D12, and compare the induced fluorescence value with the fluorescence value induced by LB and HMA to test their specificity.

Control group:

200 μL LB+1% DH5α non-transformed plasmid bacterial solution
200 μL LB+1‰ 50 mg/mL ampicillin+1% DH5αPobR^{WT} bacterial solution
196 μL LB+1‰ 50 mg/mL ampicillin+ 4 μL 50 g/L 4HB+1% DH5αPobR^{WT} bacterial solution (final concentration is 1 g/L)
196 μL LB+1‰ 50 mg/mL ampicillin+ 4 μL 50 g/L HMA+1% DH5αPobR^{WT} bacterial solution (final concentration is 1 g/L)

Experimental group (200μL system): LB+ analogs+1‰ 50 mg/mL ampicillin+1% bacterial solution (analogs include MA, HPP, Phe, Tyr, PPA, phenylethanol, phenylacetaldehyde, Trp)
Note: The concentration of the analog mother liquor used is 50 g/L

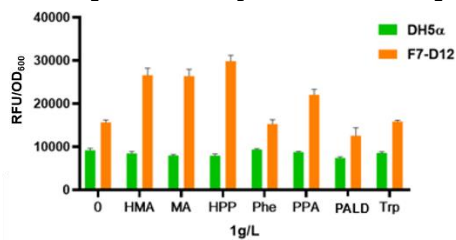

Figure 22 Induction data graph of DH5αPobR^{WT} and F7-D12 by different inducers

By comparing with the background expression, we believe that F7-D12 in this set of data still have a high response to MA and HPP.
We found that F7-D12 did not grow in 1 g/L Tyr and phenylacetaldehyde, but the fluorescence of F7-D12 with phenylacetaldehyde was extremely high.

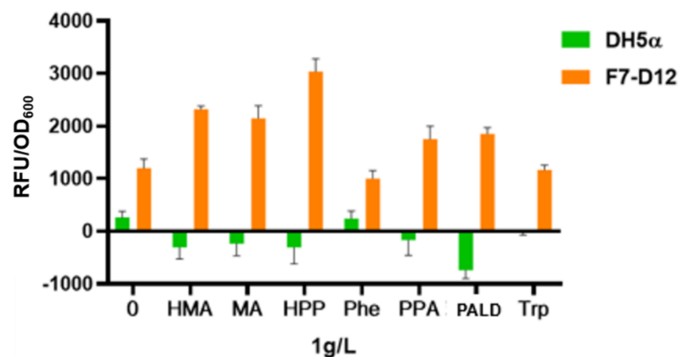

Figure 23 Induction data graph of DH5α PobR^{WT} and F7-D12 by different inducers

The enzyme labeling program of this HMA analog verification experiment was changed to Gain=80 Manual; Mirror=50% Mirror.
By comparing with the background expression, we believe that F7-D12 in this set of data still has a high response to MA and HPP.
We still found that F7-D12 did not grow in 1 g/L Tyr and phenylacetaldehyde, but the fluorescence of F7-D12 with phenylacetaldehyde was extremely high; the preliminary analysis was due to the high concentration of hydrochloric acid when Tyr used hydrochloric acid configuration, and high concentration of phenylethyl Aldehydes are harmful to cells.

7.27 Yi Dong

F4-A9 sequencing results

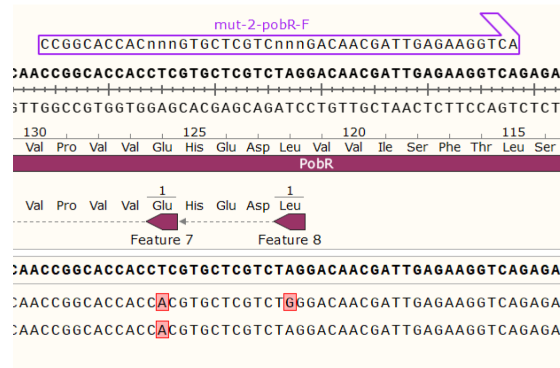

Figure 24 D12 mutation site: 122 site: Leu→Pro， 126 site: Glu→Val

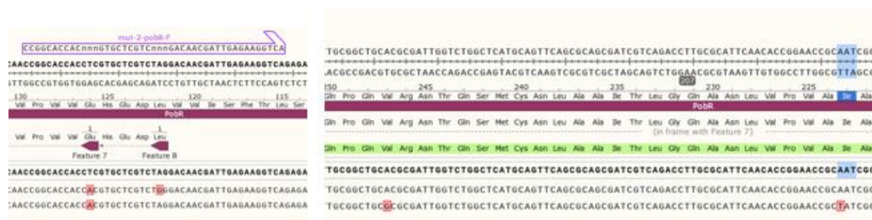

Figure 25 A9 mutation site: 126 Glu→Val; 223 Ile synonymous mutation; 247 Val→Ala