WEEK 20

8.4 Chuyao Xu

Site-directed mutagenesis of the PobR CDS

Picked 3 clones from every plate and inoculated into 5 mL LB medium and cultured at 37 ℃ for 8 h.

Then, 700 μL culture were used for storage at -20 ℃ and other cultured were store at 4 ℃ for further experiments.

8.5 Zishu Yang

Gene knockout

This week, we prepared the chemical competent cells and eletrocompetent cells of BL21*ΔtyrB* (Figure 1, 2). We successfully constructed strain BL21*ΔtyrR* and removed pTarget-tyrB from the strain. The result was proven by solid and liquid photocopying (Figure 5). Then, we tried to knock *tyrR* out based on BL21*ΔtyrB*, three times electroporation were down in total (Figure 6, 7). At the same time, gene knockout of *aspC* was prepared. pTarget-aspC (Figure 8, 9) and donor DNA overlap *aspC* were prepared (Figure 10~13).

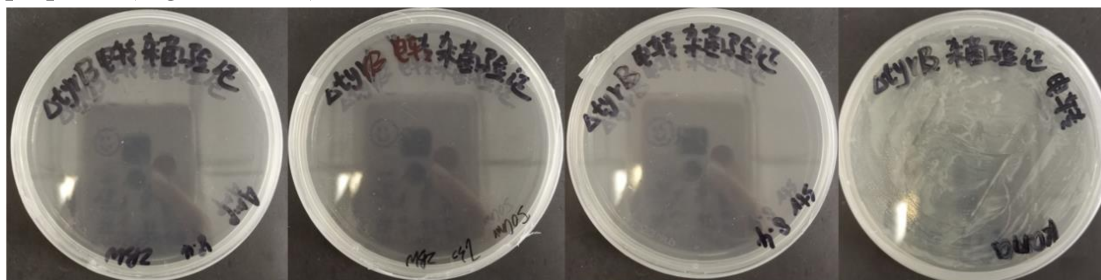


Figure 1 Pollution detection of BL21*ΔtyrB* eletrocompetent cells

Result showed that cells can only grow on the LB medium with kanamycin (with Pcas9 plasmid in the cell) in 4 kinds of LBs with different antibiotics (ampicillin, chloramphenicol, streptomycin, kanamycin).

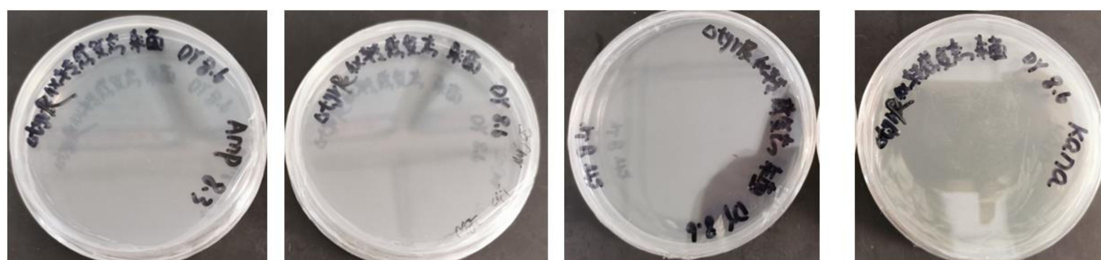


Figure 2 Pollution detection of BL21*ΔtyrB* chemical competent cells

Result showed that cells can only grow on the LB medium with kanamycin (with Pcas9 plasmid in the cell) in 4 kinds of LBs with different antibiotics (ampicillin, chloramphenicol, streptomycin, kanamycin).

Reaction system（10μL）	
tyrR-R-over-R/*tyrR*-F-over-F	0.2μL/0.2μL
2×Rapid Taq Master Mix	5μL
templete	1μL
ddw	3.6μL

Reaction progress		
98℃	2min	
98℃	30s	×30
58℃	30s	×30
72℃	1min30s	×30
72℃	5min	

Figure 3 Reaction system and reaction progress of colony PCR

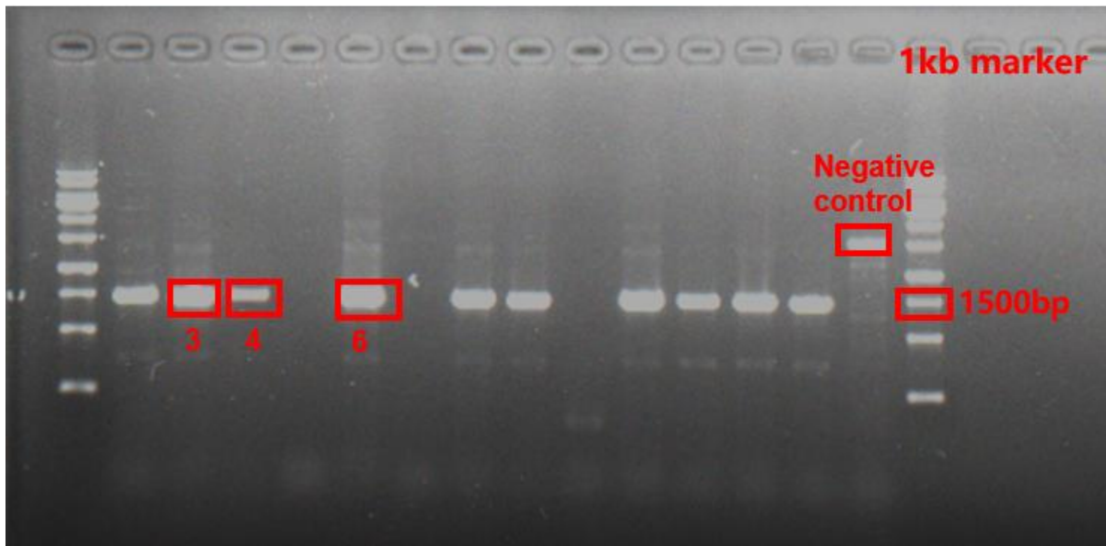


Figure 4 Agarose gel electrophoresis assay of colony PCR. *E. coli* BL21 was used as negative control

We chose clone 3 for the further experiments.

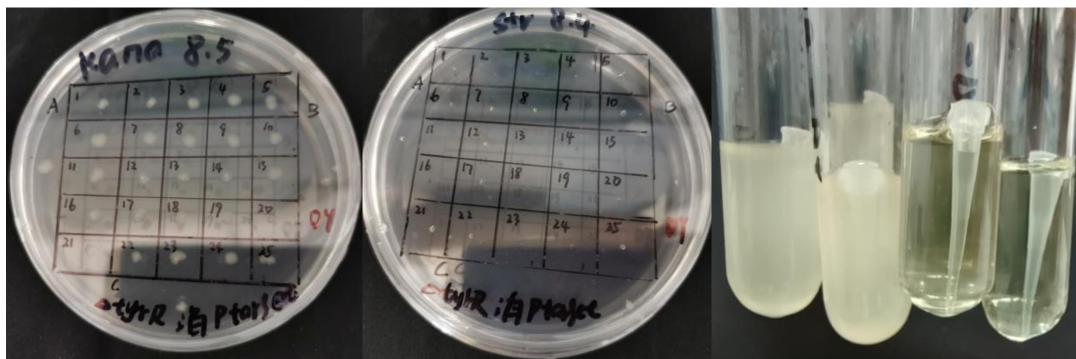


Figure 5 Solid and liquid photocopying, left, solid photocopying with kanamycin as pressure; middle, solid photocopying with streptomycin as pressure; right, liquid photocopying

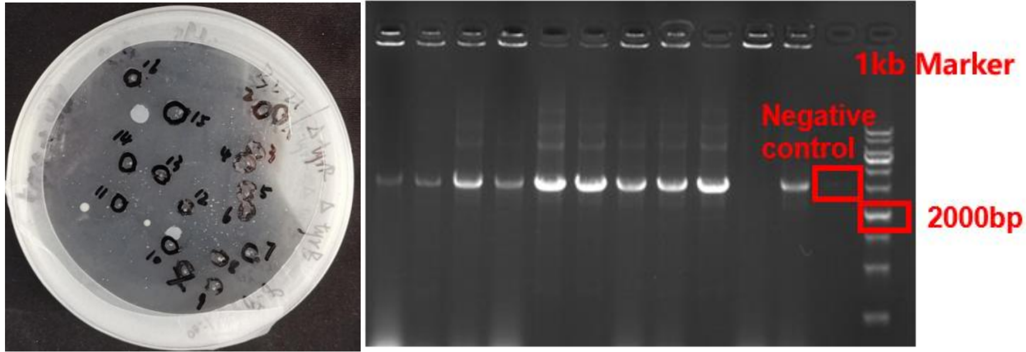


Figure 6 Electroporation result of *tyrR* knockout out based on BL21*ΔtyrB* (1$_{st}$ time)
11 clones were picked to having colony PCR.

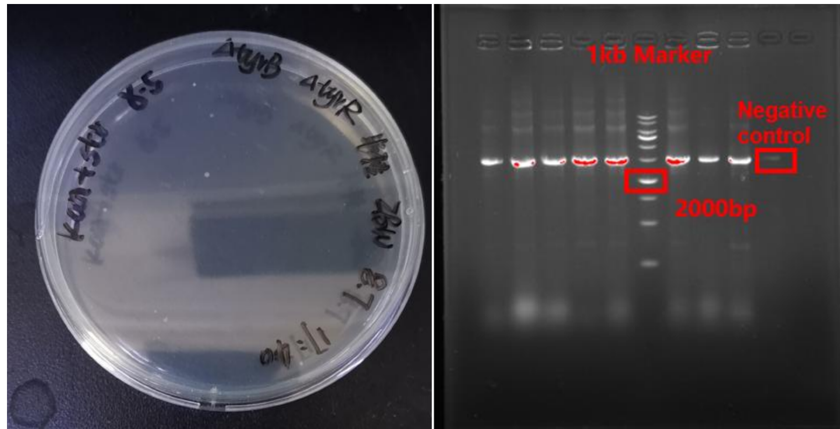


Figure 7 Electroporation result of *tyrR* knockout out based on BL21*ΔtyrB* (2$_{nd}$ time)
8 clones were picked to have colony PCR.

No correct band (1400 bp) was shown in two times colony PCR. Therefore, *tyrR* knockout failed in two times. Then we tried to optimize electroporation method and increased the concentration of donor DNA *tyrR*-overlap to have *tyrR* knockout again.

Reaction system（50μL)	
pTarget-*aspC*-F/ pTarget-*aspC*-R	1μL/1μL
2× HF Mix	25μL
pTarget 613	1μL
ddw	22μL

Reaction progress		
98℃	2min	
98℃	30s	×30
53-56℃	30s	×30
72℃	1min50s	×30
72℃	5min	

Figure 8 PCR reaction system and reaction progress for preparing pTarget-aspC

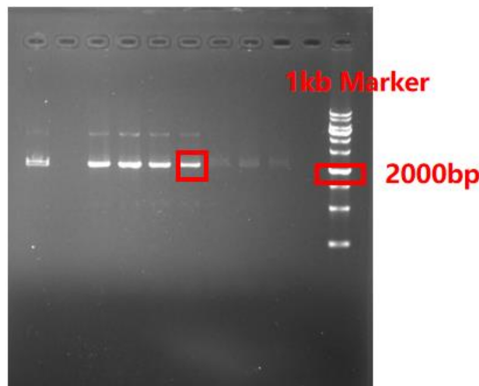


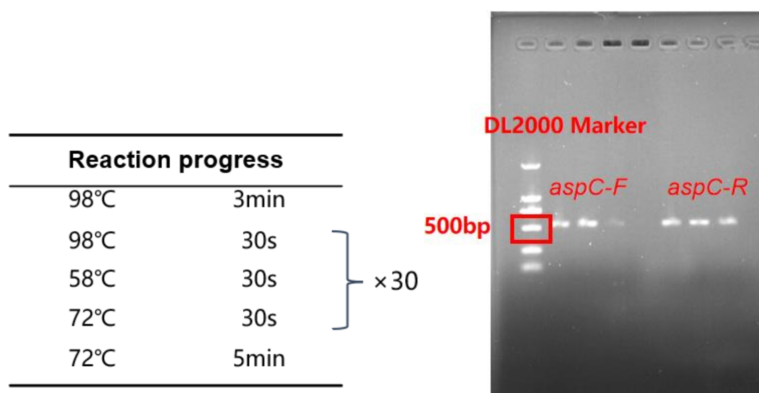
Figure 9 Agarose gel electrophoresis assay of PCR for pTarget-aspC

We extracted the target DNA from gel because the extra unwanted DNA fragments were produced in PCR progress (concentration of target DNA after gel extraction: 51.065 ng/μL).

Reaction system (50μL)	
aspC-F-Over-R/*aspC*-F-Over-F	1μL/1μL
2×HF Mix	25μL
BL21genome	1μL
ddw	22μL

Reaction system (50μL)	
aspC-R-Over-R/*aspC*-R-Over-F	1μL/1μL
2×HF Mix	25μL
BL21genome	1μL
ddw	22μL

Figure 10 PCR system for amplify *aspC*-F & *aspC*-R



Reaction progress		
98℃	3min	
98℃	30s	×30
58℃	30s	
72℃	30s	
72℃	5min	

Figure 11 PCR for amplify *aspC*-F & *aspC*-R. Left, reaction progress; right, Agarose gel electrophoresis assay, bands correct (500 bp)

Reaction system (10μL)	
aspC-F-Over-F/*aspC*-R-Over-R	1μL/1μL
aspC-F/*aspC*-R	2μL/2μL
2×HF Mix	25μL
ddw	19μL

Reaction progress		
98℃	3min	
98℃	30s	×30
55℃	30s	
72℃	1min	
72℃	5min	

Figure 12 Overlap ligation system and progress for donor DNA overlap *aspC*

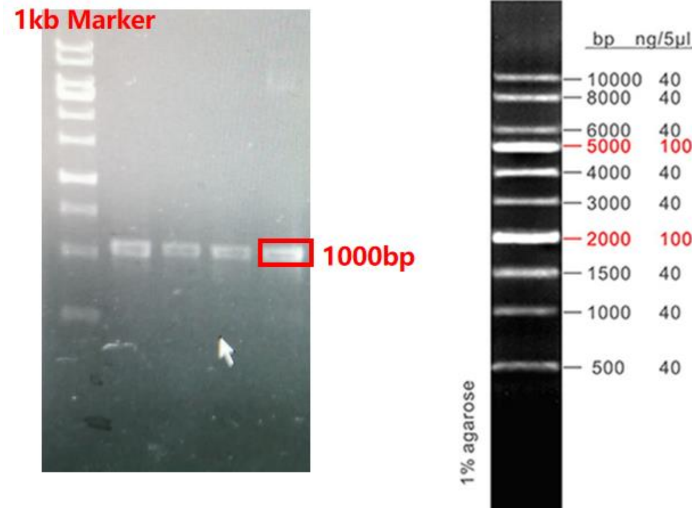


Figure 13 Agarose gel electrophoresis assay of overlap ligation, bands are correct (1000 bp)

8.5 Baitong Shen

Site-directed mutagenesis of the PobR CDS

Extracted plasmid DNA from the culture at 4℃ and send them to sequencing.

8.6 Peng Jiang

Modeling and docking

Submit the Sequence

Using protein simulation tool RoseTTAFold web (https://robetta.bakerlab.org/) to submit PobR wild-type animo acid sequence, at the same time try to install and run in the laptop RoseTTAFold, database download in mobile hard disk. RoseTTAFold link (https://github.com/RosettaCommons/RoseTTAFold).

8.7 Chao Chen

Re-screening.

We stabilized the system and screened the 647 clones selected last time (Figure 14), then we re-screened the clones (83 clones in total) which induction intensity of HMA is higher than the induction intensity of 4HB to DH5αWT (about 800) (Figure 15~18).

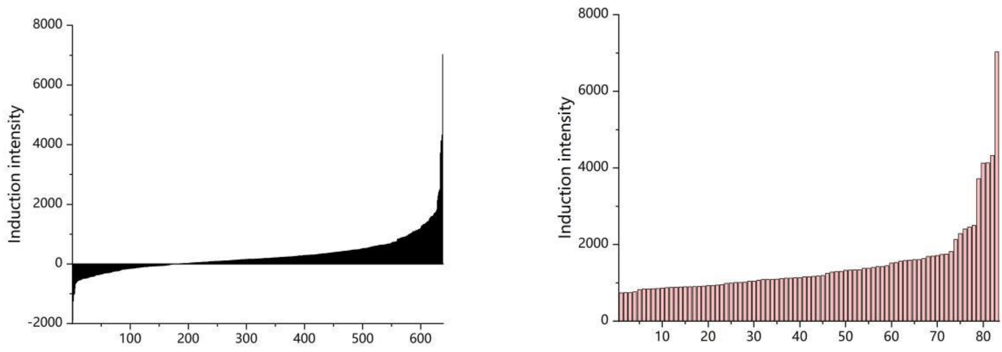


Figure 14 Left, 647 clones' induction intensity of HMA; right, 83 clones' induction intensity of HMA which were higher than 800

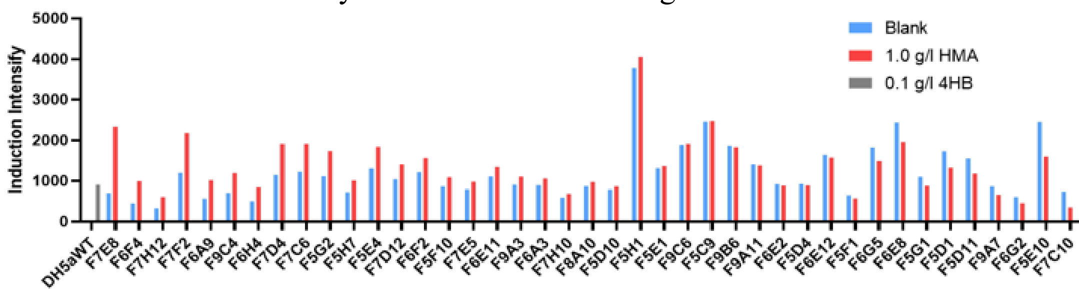


Figure 15 Part of 83 clones' induction intensity in re-screening

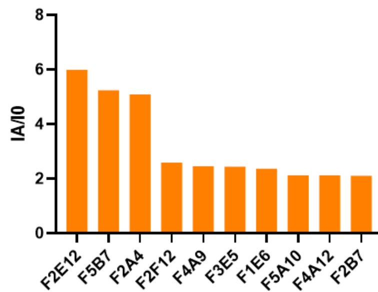
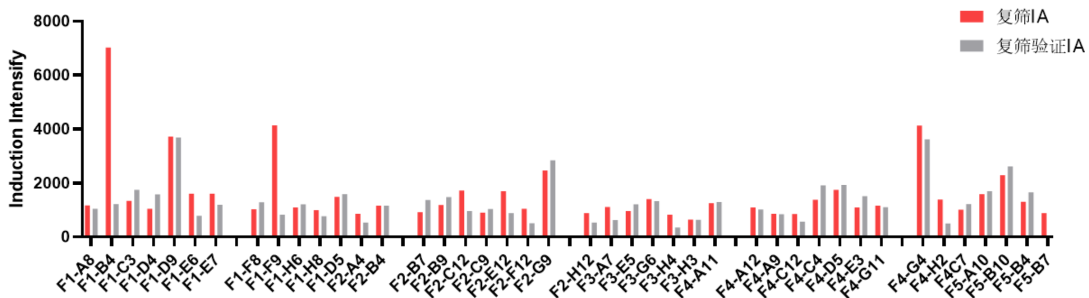


Figure 16 10 clones' ratio of IA/I0 which were highest in 83 clones



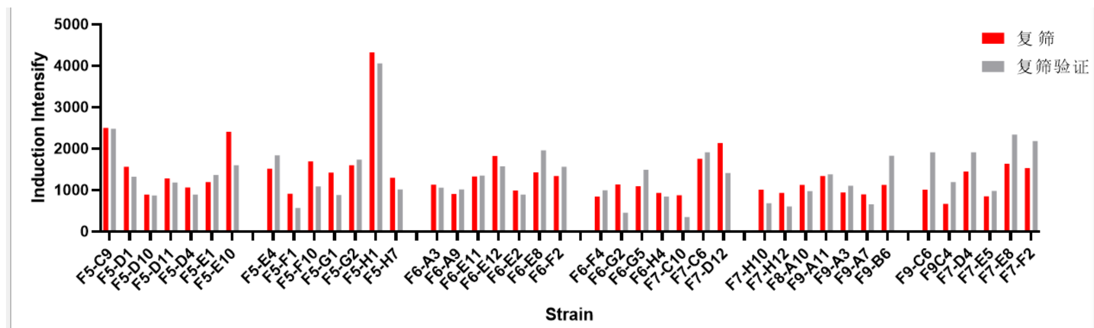


Figure 17 Comparison of 83 clones' IA in screening and re-screening, from the result we found that the data stability is good and the experiments have repeatability

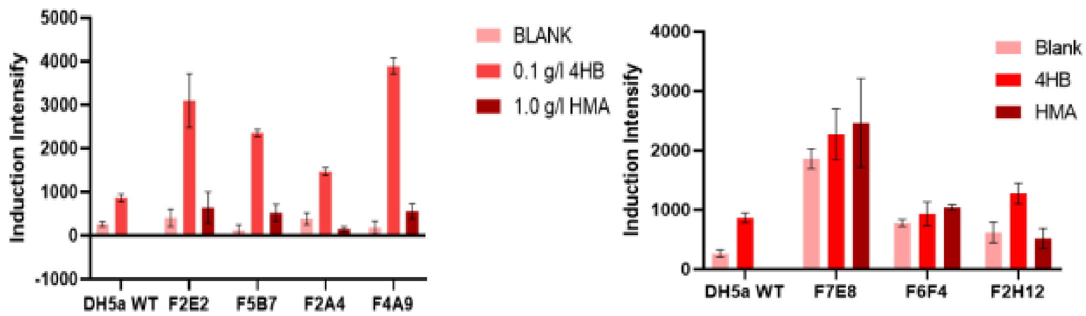


Figure 18 Repetition of the re-screening

The induction intensity of clones with the highest ratio of IA/I0 in re-screening last time. Figure 18 left showed the induction intensity of clones with the highest ratio of IA/I0 in the first plate of re-screening last time; Figure 18 right showed the induction intensity of clones with the highest ratio of IA/I0 in the second plate of re-screening last time. Compared results in 2 times, we found that the data of repetition do not have repeatability. We suspected the culture was polluted during storage. Therefore, streak plate method was used to isolated monoclones of F5-B7 C7/C9 and F4-A9 C10/C11.

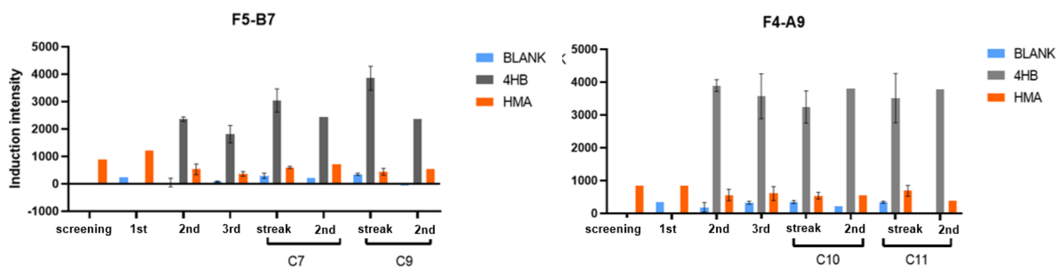


Figure 19 Screening results after isolating monoclones compared with data before, left, F5-B7; right, F4-A9

After analyzes the result we found that the IA is stayble in these experiments. Therefore, F5-B7 and F4-A9 were believed to have the ability to be induced by HMA.

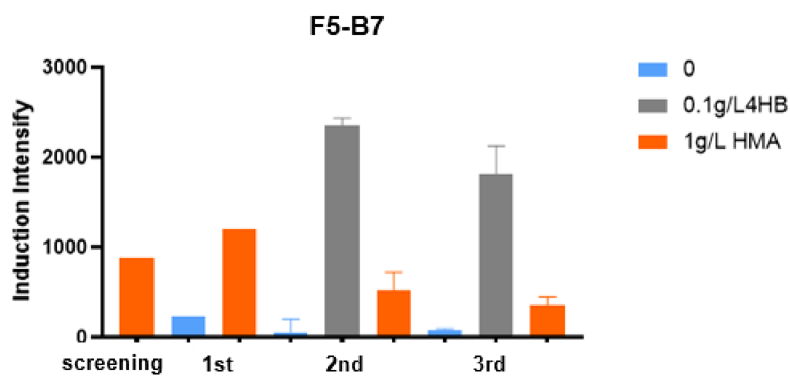


Figure 20 Data summarization of F5-B7

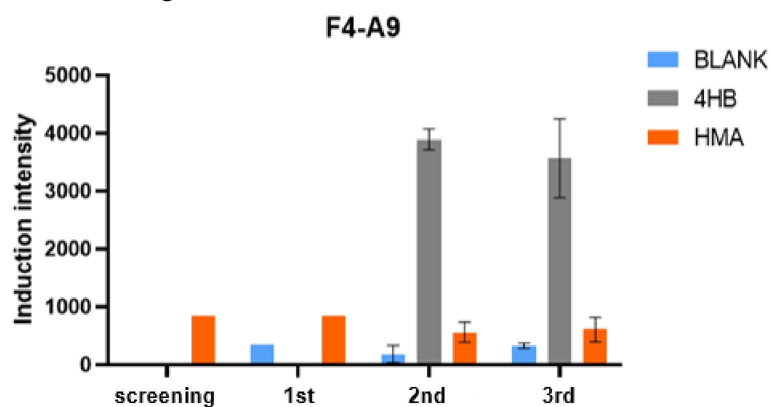


Figure 21 Data summarization of F4-A9

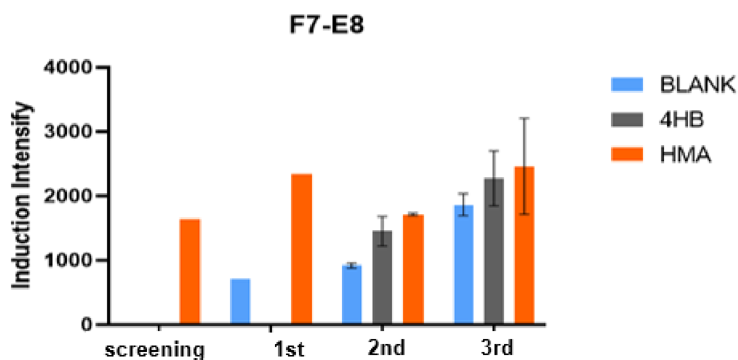


Figure 22 Data summarization of F7-E8

8.7 Xinlu Liu

Site-directed mutagenesis of the PobR CDS

1. Sequencing result:

Spm126/spm122 have expected mutation on the plasmids, spm247 does not.

2. Site-directed mutagenesis PCR (50 μL×1)

Reaction system:

247spm
247spm-F: 1 μL
247spm-R: 1 μL
pYB1a-PobR-eGFP-Cmr: 1 μL
2×HF Mix: 25 μL

DDW: 22 µL

Reaction procedure

98 ℃ 5 min

98 ℃ 30 s ⎫
60 ℃ 30 s ⎬ ×24
72 ℃ 3 min ⎭

72 ℃ 5 min

16 ℃ 1 h

Agarose gel electrophoresis assay showed correct bands, the concentration of the product after purified:

247spm (PCR): 38 ng/µL

3. Transformation

System:

	Spm247
Competent cells	DH5α: 100 µL
Digestion product	1 µL (38 ng)
plate	Amp50

Culture at 37℃ for 12 h.

8.8 Shuning Guo

Site-directed mutagenesis of the PobR CDS

Picked 2 clones from the plate and inoculated into 5mL LB medium and culture at 37℃ for 8 h.

Then, 700 µL culture were used for storage at -20℃ and other culture were store at 4℃ for further experiments.

8.9 Peiyao Chang

Chloramphenicol ALE pre-experiment

Transformed pYB1a-PobR$^{F4\text{-}A9}$-eGFP-Cmr and pYB1a-PobR$^{F7\text{-}D12}$-eGFP-Cmr into BL21 competent cells respectively.

Experimental system as below:

Control group: 200μl LB；200μl LB+1%F7-D12 (BL21)+2μl Amp

Experimental group: prepare Chloramphenicol of 10、20、30、40、50、60、70、80、90、100mg/mL

x3 ⎧ 200μl LB+1% F7-D12 (BL21) +2μl Amp+2μl different concentration of Chloramphenicol
⎩ 200μl LB mixture **(contain1.0 g/L HMA)** +1% F7-D12 (BL21) +2μl Amp +2μl different concentration of Chloramphenicol

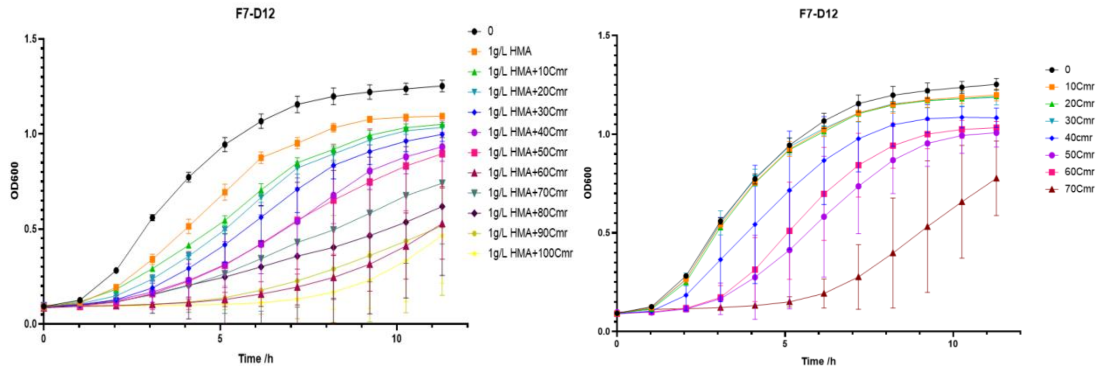


Figure 23 Growth curve of F7-D12 under the pressure of different concentrations of chloramphenicol, left, added 1 g/L HMA as inducer; right, no inducer

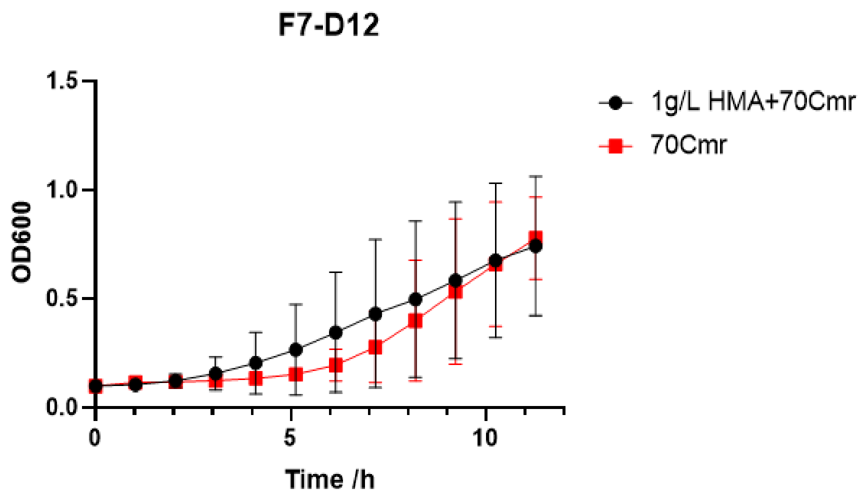


Figure 24 Comparation of the growth curve of F7-D12 with or without inducer under the pressure of 70 μg/mL chloramphenicol

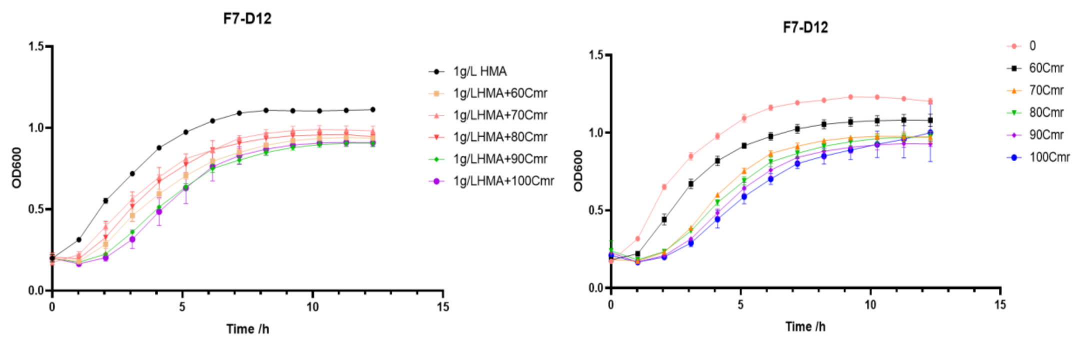


Figure 25 Growth curve of F7-D12 under the pressure of different concentrations of chloramphenicol, left, added 1 g/L HMA as inducer; right, no inducer in the second time

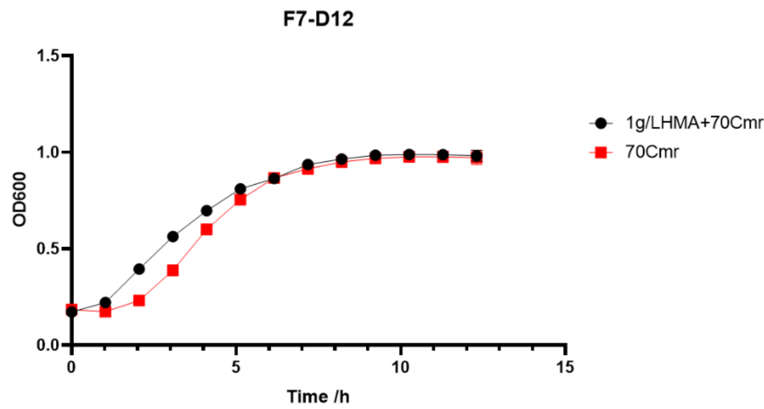


Figure 26 Comparation of the growth curve of F7-D12 with or without inducer under the pressure of 70 μg/mL chloramphenicol in the second time.

Growth curve of F7-D12 (BL21) showed remarkable difference in two experiments. Therefore, the stability of the data needs to be improved.

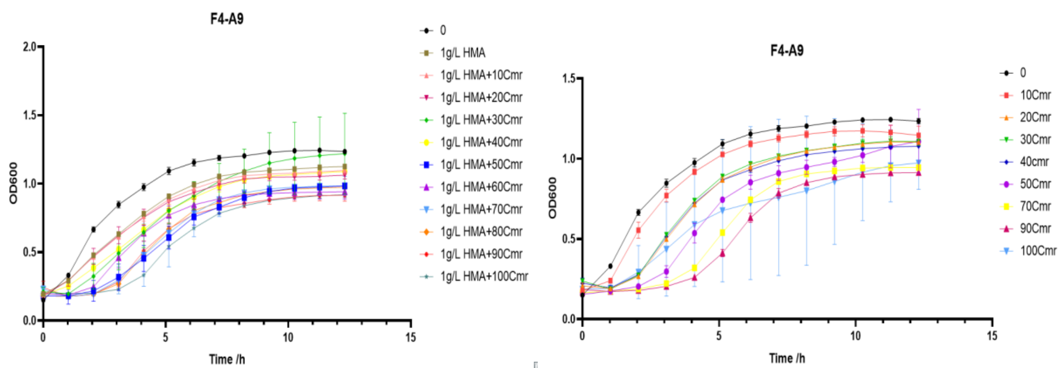


Figure 27 Growth curve of F4-A9 under the pressure of different concentrations of chloramphenicol, left, added 1 g/L HMA as inducer; right, no inducer in the second time

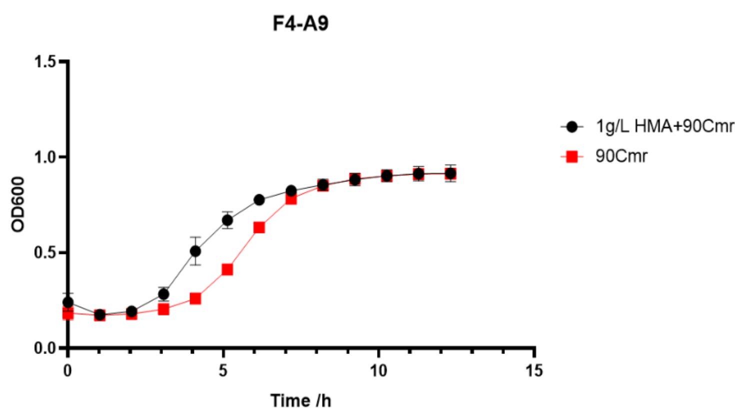


Figure 28 Comparation of the growth curve of F4-A9 with or without inducer under the pressure of 90 μg/mL chloramphenicol

The growth curve of BL21 F4-A9 with or without inducer only showed differences under the pressure of 90 μg/mL chloramphenicol. This was out of line with expectations because F4-A9 was expected to show a more strongly growth

suppression under the pressure of same concentration chloramphenicol compared with F7-D12 because its low background expression. Therefore, we designed to do experiment on the growth curve of DH5αWT under the pressure of different concentrations of chloramphenicol add or do not add HMA.

8.9 Beimeng Zhang

Site-directed mutagenesis of the PobR CDS

Extracted plasmid DNA from the culture at 4 ℃ and send them to sequencing.

8.10 Baitong Shen

Site-directed mutagenesis of the PobR CDS

1. Sequencing result:

Both the two clones do not have expected mutation on the plasmids.

2. DpnI digestion

Reaction system:

Spm247
DpnI: 0.2 μL
10×Cutsmart: 1 μL
247spm (PCR) (38 ng/μL): 8.8 μL
DDW: 0

Reaction procedure:

37 ℃ 2 h

70 ℃ 20 min