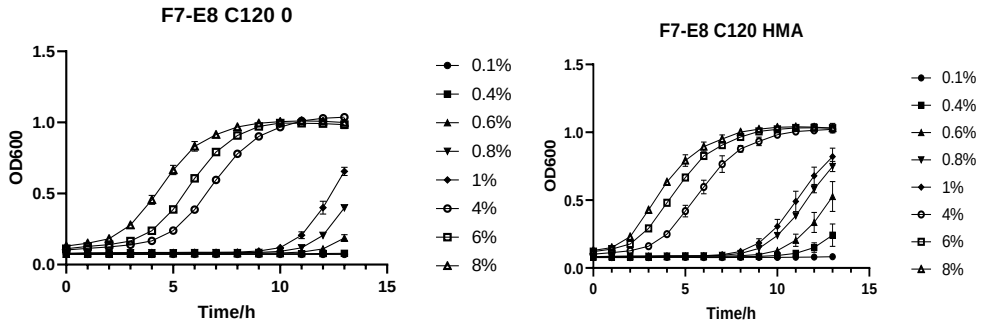
# WEEK 24

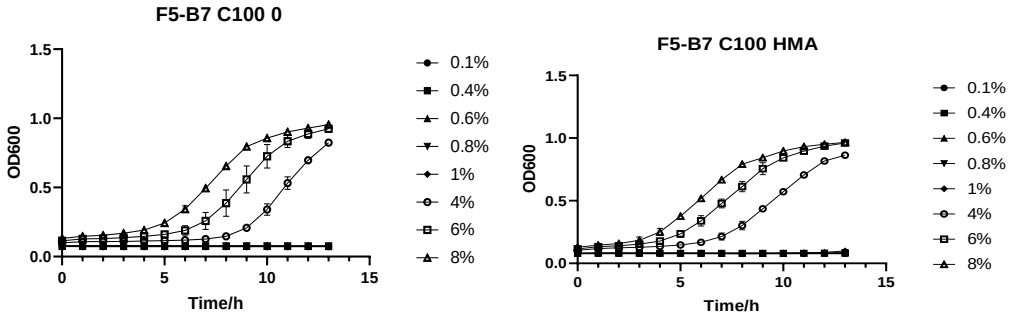
## 9.9 Yulong Zhang

**Adaptive evolution experiment under chloramphenicol pressure**

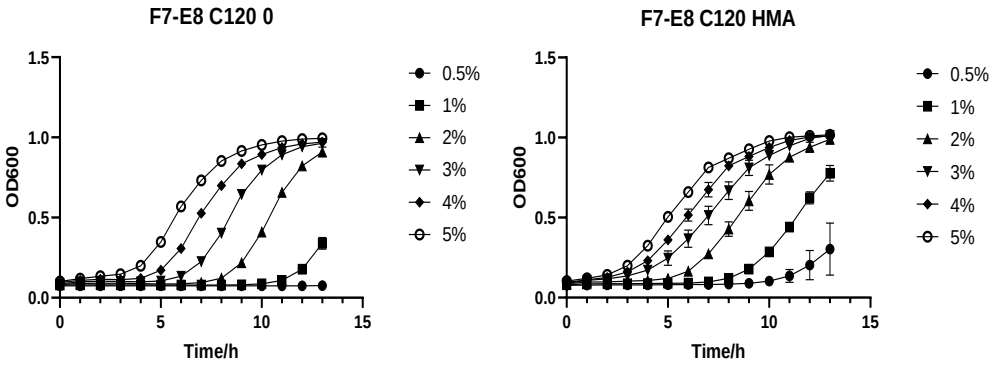
This week, we first carried out the inoculation amount gradient experiment under chloramphenicol pressure to determine the appropriate inoculation amount. Under this inoculation amount, there was a great difference between the growth of uninduced strains and that of induced strains (Figure 1). According to the experiment, the adaptive evolutionary pressure experiment was carried out under 3% bacteria solution, and then the bacteria F7-E8 and F5-B7 (BL21) with better induction degree were screened out and repeated chloramphenicol was carried out for the adaptive pressure experiment.



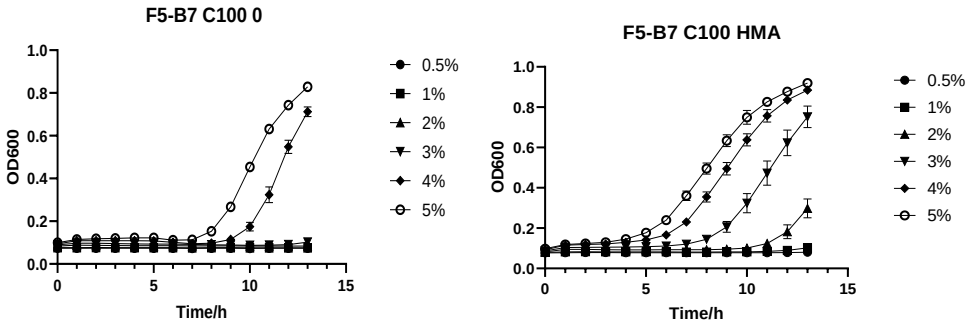

（1） growth curve of BL21 F7-E8 in uninduced and induced state at 120 chloramphenicol solution pressure (8.30)



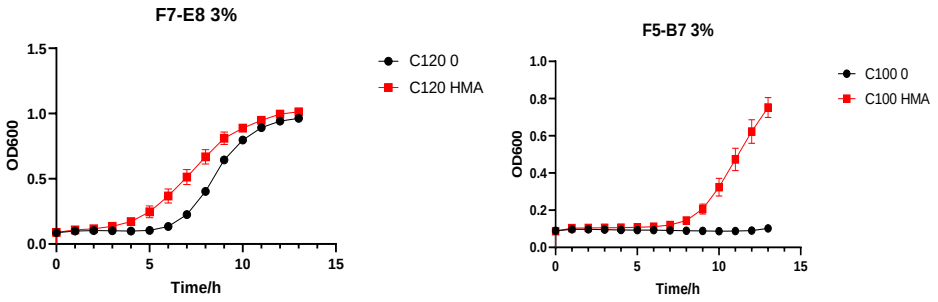

（2） growth curve of BL21 F5-B7 in uninduced and induced state at 100 chloramphenicol solution pressure (8.30)




（3） growth curve of BL21 F7-E8 in uninduced and induced state at 120 chloramphenicol solution pressure (8.31)




（4） growth curve of BL21 F5-B7 in uninduced and induced state at 100 chloramphenicol solution pressure (8.31)




（5） growth curve of BL21 F7-E8 and BL21 F5-B7 under 120 mg/ mL chloramphenicol and 100 mg/ mL chloramphenicol solution pressure inoculated with 3 % HMA and without HMA induction, respectively

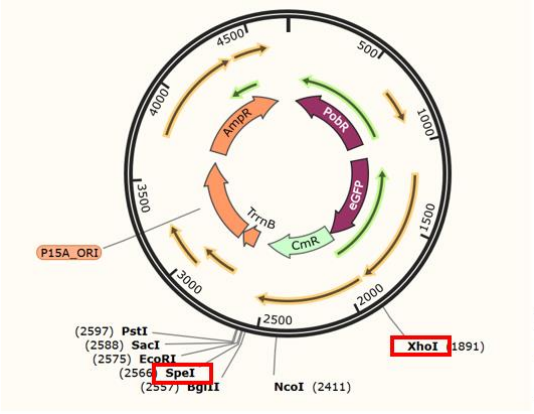
Figure 1 Growth curve of uninduced strains and induced strains under chloramphenicol pressure

## 9.11 Jiameng Nie

**pYB1a-PobR$^{mut}$(F5-B7)-eGFP-SacB constructing experiment**

pYB1a-PobR$^{mut}$(F5-B7)-eGFP-SacB was constructed in order to directionally evolve

the HmaS enzyme to obtain the mutant HmaS enzyme that can efficiently transform HPP into HMA.



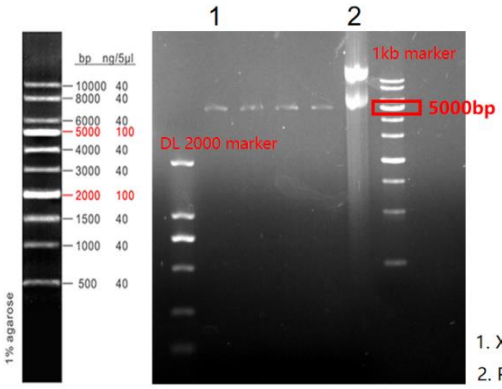

PYB1a-PobR$^{mut}$-eGFP-Cmr : 4710bp
XhoI-SpeI (Cmr) : 675bp
XhoI-SpeI (template) : 4035bp

Figure 2 pYB1a-PobR$^{mut}$-eGFP-Cmr plasmid map

| Reaction system (50μL×4) | |
|---|---|
| PYB1a-PobR$^{mut}$-eGFP-Cmr | 3μL (1μg) |
| XhoI | 1μL |
| SpeI | 1μL |
| rCutsmart | 5μL |
| DDW | 40μL |

| Reaction procedure | |
|---|---|
| 37℃ | 2h |

Figure 3 XhoI, SpeI double enzyme digestion system and procedure




1. XhoI, SpeI double enzyme
2. PYB1a-PobR$^{mut}$-eGFP-Cmr original template

Figure 4 XhoI, SpeI double enzyme digestion electrophoresis results

A single 5000 bp-6000 bp band was found in the result of double enzyme digestion, but no 700 bp band was found. It was speculated that only one end was cut.

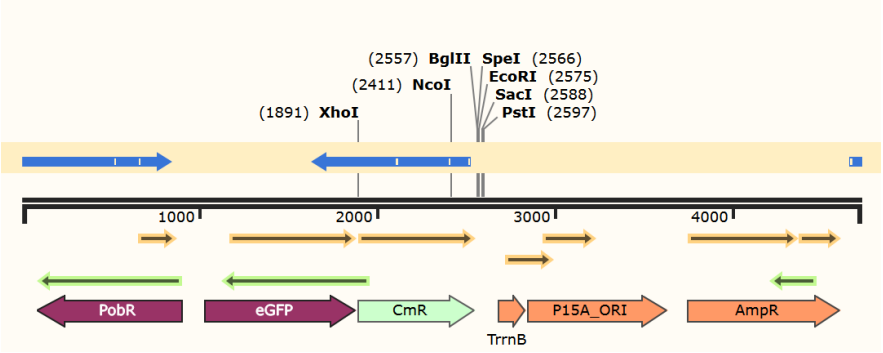


Figure 5 pYB1a-PobR$^{mut}$(F5-B7)-eGFP-Cmr sequencing results（POBR-TOP-F/ CMR-R)

The sequencing results proved that the XhoI site was correct, but the SpeI site was not detected, so a single enzyme digestion was attempted for verification.

| Reaction system（20μL） | |
|---|---|
| PYB1a-PobR$^{mut}$-eGFP-Cmr | 1.5μL (0.4μg) |
| XhoI/SpeI | 0.4μL/0.4μL |
| rCutsmart | 2μL |
| DDW | 15.7/16.1 μL |

| Reaction procedure | |
|---|---|
| 37℃ | 2h |
| 80℃ | 20min |

Figure 6 XhoI, SpeI double digestion and single digestion verification system and procedure

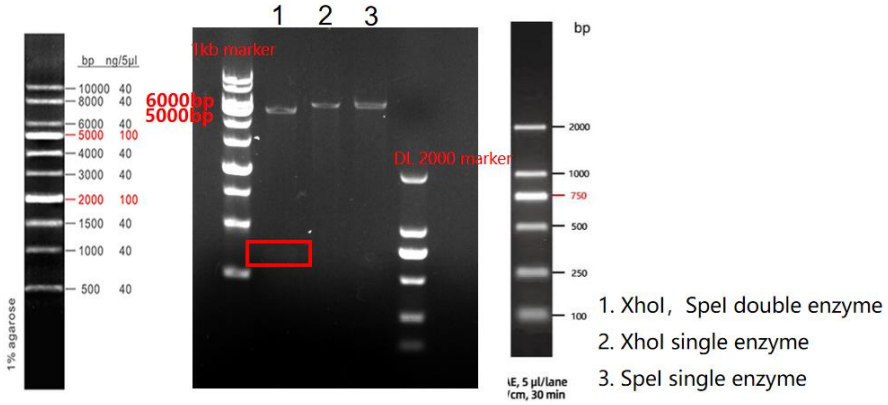



Figure 7 XhoI, SpeI double digestion and single digestion verification electrophoresis results

The results showed that a similar band appeared in all the single enzyme digestion, and a similar band appeared in the double enzyme digestion about 5000 bp, which was suspected to be about 750 bp.

After the enzyme digestion verification was correct, considering that the results were blurred because the bands were too shallow, the sample loading was increased for observation and enzyme digestion was carried out again.

| Reaction system（50μL×4） | |
|---|---|
| PYB1a-PobR$^{mut}$-eGFP-Cmr | 2.8μL (937ng) |
| XhoI | 1μL |
| SpeI | 1μL |
| rCutsmart | 5μL |
| DDW | 39.7μL |

| Reaction procedure | |
|---|---|
| 37℃ | 2h |

Figure 8 XhoI, SpeI double enzyme digestion carrier system and procedure

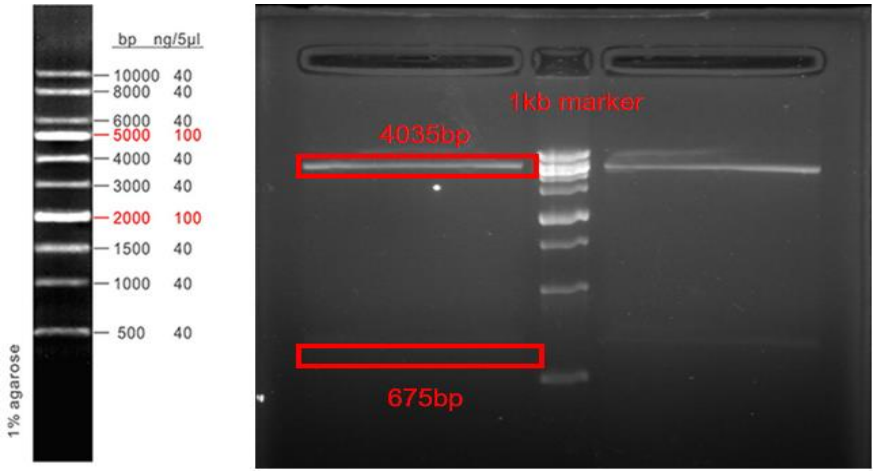



Figure 9 XhoI, SpeI double enzyme digestion electrophoresis results

The enzyme was cut correctly, and the gel was cut and recycled (four in one) at a concentration of 6 ng/μL.

| Reaction system (50μL×6) | | Reaction procedure | | |
|---|---|---|---|---|
| PK18mob-SacB | 1μL | 95℃ | 5min | |
| SacB-Spe I -R | 1μL | 95℃ | 30s | ×30 |
| SacB-Xho I -F | 1μL | 55/56℃ | 30s | |
| 2×HF Mix | 25μL | 72℃ | 1min30s | |
| DDW | 22μL | 72℃ | 5min | |

Figure 10 SacB fragment pcrsystem and procedure

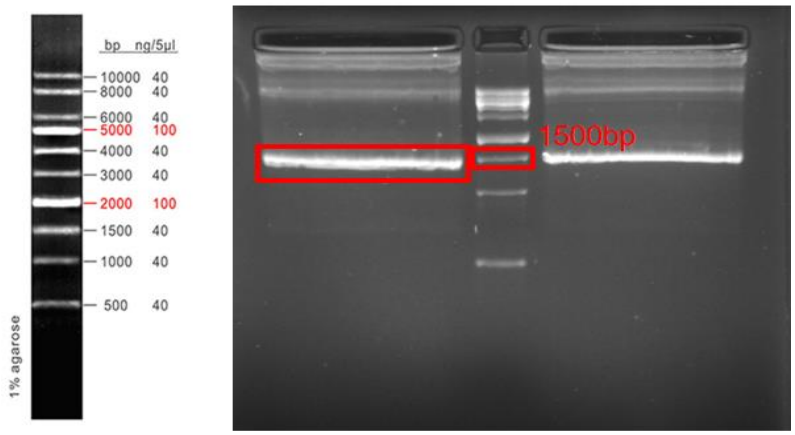



Figure 11 SacB fragment gel recovery detection results

The PCR product was four tubes in one, and the recovery concentration was 44.769 ng/μL.

| Reaction system（50μL×2） | |
|---|---|
| sacB(PCR切胶) | ①15μL(68ng/μL)<br>②20μL(51ng/μL) |
| Xho I | 1μL |
| Spe I | 1μL |
| rcutsmart | 5μL |
| ddw | 28μL |

Figure 12 SacB fragment enzyme digestion system

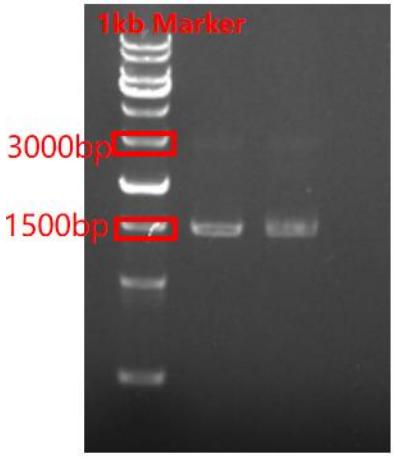



Figure 13 SacB fragment detection result

The fragment size was correct, and the gel was cut and recycled (four in one). The concentration was 11 ng/μL.

| connection system（10μL×3） | |
|---|---|
| SacB（Gel recovery） | 6μL(11ng/μL) |
| PYB1a-PobRmut-eGFP | 2.9μL(6ng/μL) |
| T4 ligase | 0.1μL |
| 10×Buffer | 1μL |

- conversion(×4):
  10μLproduct+100μLDH5α chemoreceptive state
- Ice bath (30mins)、Heat shock (45s) in 42℃、
  Ice bath (5mins)
- Add LB 500μL、Resuscitate for 1h in 37℃
- 4000g centrifuge for 3mins、coat all（Amp）、
  37℃ 12h

Figure 14 connection system and transformation system

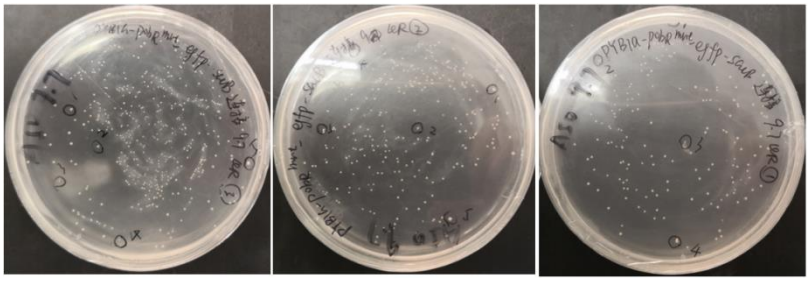


Figure 15 plates growth results after transformation

The growth of the three plates was good, and 5 single colonies were selected from each plate for colony PCR.


| Reaction procedure | | |
|---|---|---|
| 98℃ | 3min | |
| 98℃ | 30s | ×30 |
| 48℃ | 30s | |
| 72℃ | 1min | |
| 72℃ | 5min | |

Figure 16 Colony PCR result

Colony PCR was correct.

## 9.12 Jiameng Nie

**pRB1s-HmaS generation of mutant library replacing with anderson promoter**

pRB1s-HmaS generation of mutant library replacing with anderson promoter, the pRB1s-Anderson-HmaS plasmid was successfully constructed, the correct plasmid has been transferred to the BW-BL21 receptive state, and tried to use SDS-PAGE to detect protein expression.

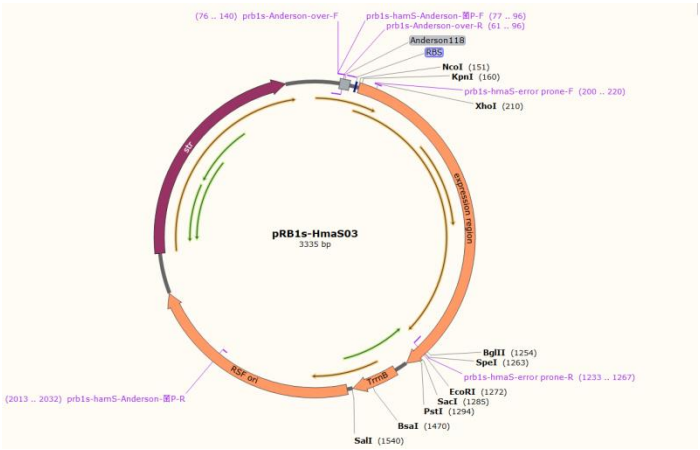



Figure 17 PRB1s-Anderson-hmaS plasmid

| Reaction system（50μL） | |
|---|---|
| *Anderson-over-F / Anderson-over-R* | 1μL/1μL |
| template(181 ng/μL) | 1μL |
| 2×HF Mix | 25μL |
| ddw | 22μL |

| Reaction procedure | | |
|---|---|---|
| 98℃ | 5min | |
| 98℃ | 30s | ×30 |
| 55-70℃ | 30s | |
| 72℃ | 3min 20s | |
| 72℃ | 5min | |

Figure 18 PCR reaction system

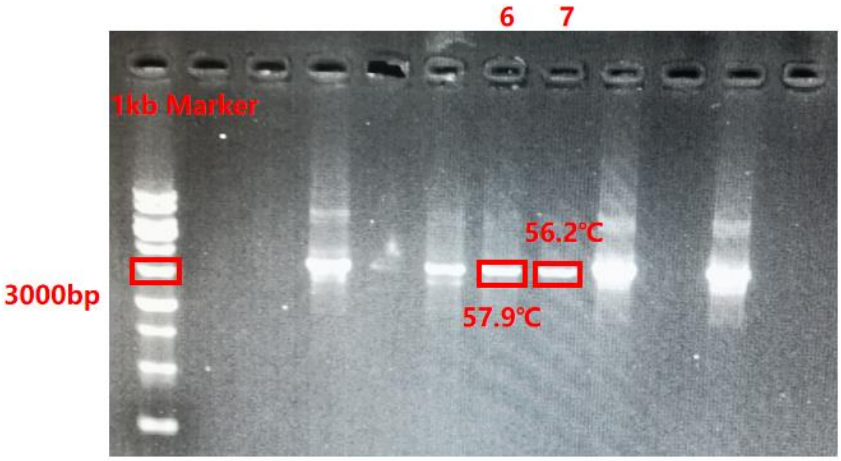



Figure 19 Overlap PCR detection result

Electrophoresis results showed that there was no heteroband, and then the products were purified at 62 ng/μL and 72 ng/μL, respectively. Finally, the products were digested by DpnI and transformed.

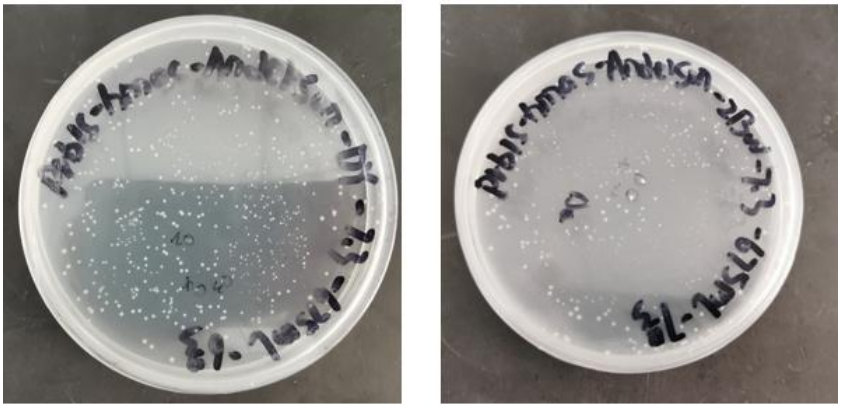


Figure 20 Plates growth results

Pick and shake bacteria from two plates.

- **reaction system (10μL)**
- DNA: 2μl (180ng)
- SpeII-HF: 0.2μl
- rCutsmart:1μl
- CIP: 0.2 μl
- DDW: 6.6 μl


Figure 21 enzyme digestion system, electrophoresis result

The bands were correct and the final verification of sequencing was carried out.

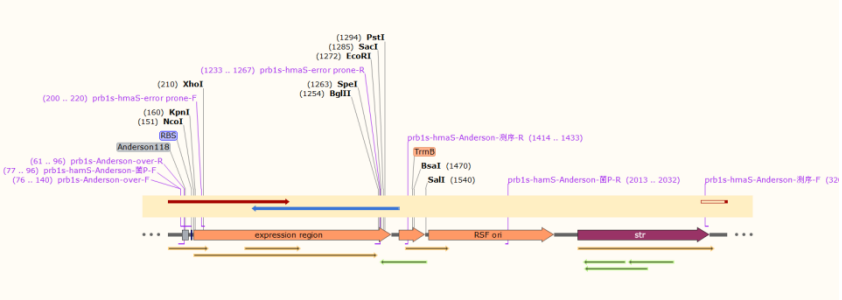



Figure 22 pRB1s-Anderson-HmaS sequencing results

Sequencing comparison proved that the Anderson promoter was successfully constructed and the arabinose promoter was eliminated.

Transfer the correct plasmid into BW/BL21 receptive state.

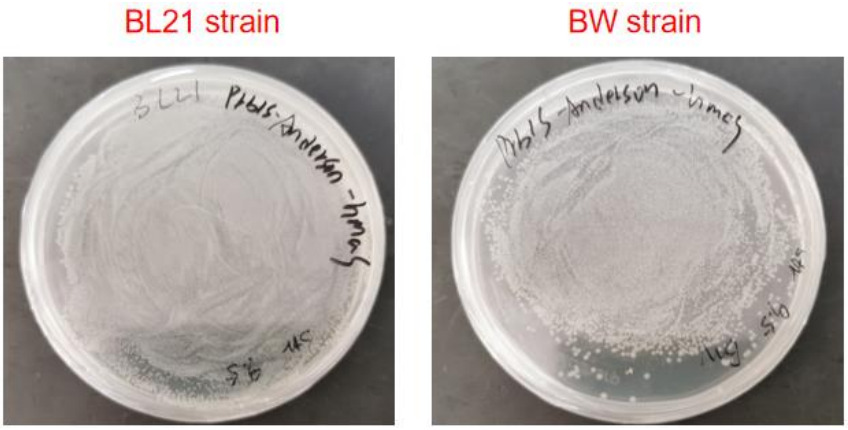



Figure 23 plates growth results

Monoclonal clones were selected and inoculated to preserve the bacteria. SDS-PAGE was performed after inoculation.

## 9.13 Zishu Yang

### pLB1s-HmaS-aroG$^{fbr2}$-pheA$^{fbr2}$ constructing experiment

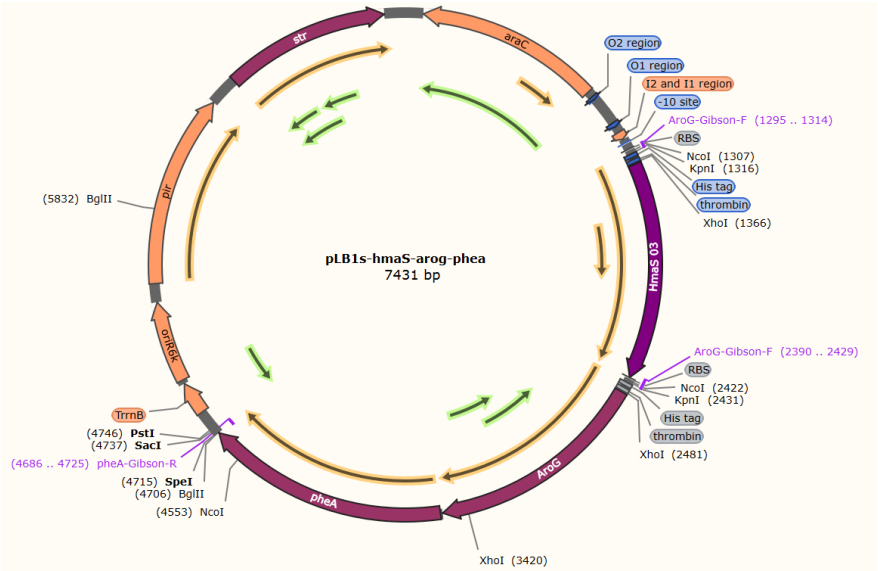


Figure 24 pLB1s-hmaS-aroGfbr2-pheAfbr2 plasmid map

| Reaction system (50μL) | |
|---|---|
| *aroG-Gibson*-F/*pheA*-Gibson-R | 1μL/1μL |
| PYB1a-aroG$^{fbr2}$-phe$^{fbr2}$ | 1μL |
| 2×HF Mix | 25μL |
| ddw | 20μL |

| Reaction procedure | | |
|---|---|---|
| 98℃ | 3min | |
| 98℃ | 30s | ×30 |
| 60℃ | 30s | |
| 72℃ | 1min15s | |
| 72℃ | 10min | |

Figure 25 *aroG$^{fbr2}$-phe$^{fbr2}$* fragment preparation PCR system and procedure (×2)

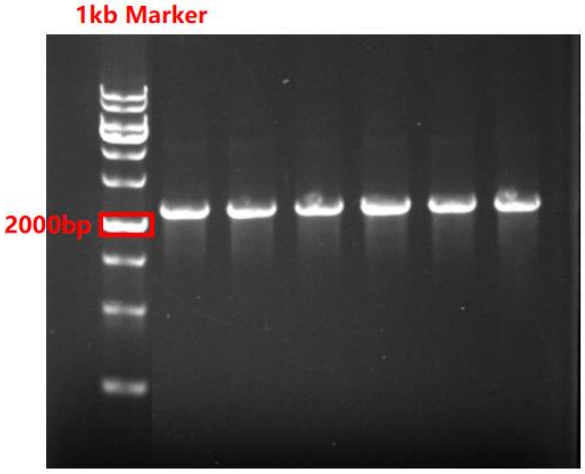



Figure 26 electrophoresis results

The 2296 bp fragment was correct with no heterozygots, and the product was purified (two-in-one) at a concentration of 132 ng/uL.

| Reaction system (50μL) | |
|---|---|
| *HmaS03-Gibson*-F/R | 1μL/1μL |
| *PLB1s-hmas-tktA-ppsA* | 1μL |
| 2×HF Mix | 25μL |
| ddw | 20μL |

| Reaction procedure | | |
|---|---|---|
| 98℃ | 3min | |
| 98℃ | 30s | ×30 |
| 60℃ | 30s | |
| 72℃ | 50s | |
| 72℃ | 10min | |

Figure 27 pLB1s-HmaS carrier preparation PCR system and procedure (×2)

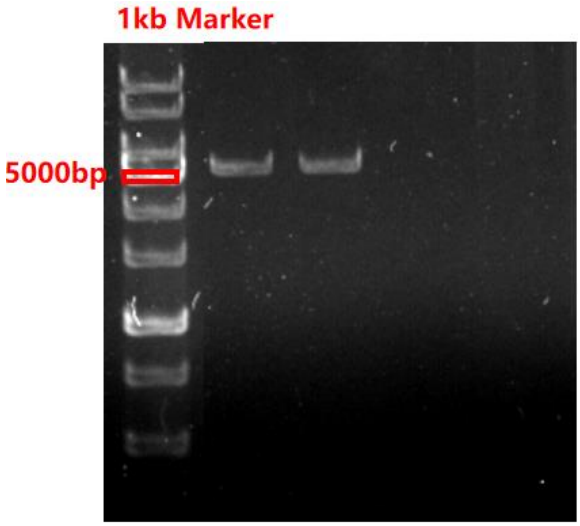



Figure 28 electrophoresis results

The carrier band was 5135 bp, and the product was purified (two-in-one) at a concentration of 343 ng/uL.

| Reaction system (20μL) | |
|---|---|
| PLB1s-hmas | 2.5uL |
| aroG$^{fbr2}$-phe$^{fbr2}$ | 1.8μL |
| 5×ceⅡ Buffer | 4μL |
| ExnaseⅡ | 2uL |
| ddw | 9.7μL |

| Reaction procedure | |
|---|---|
| 37℃ | 30min |

Figure 29 Gibson connection system and procedure

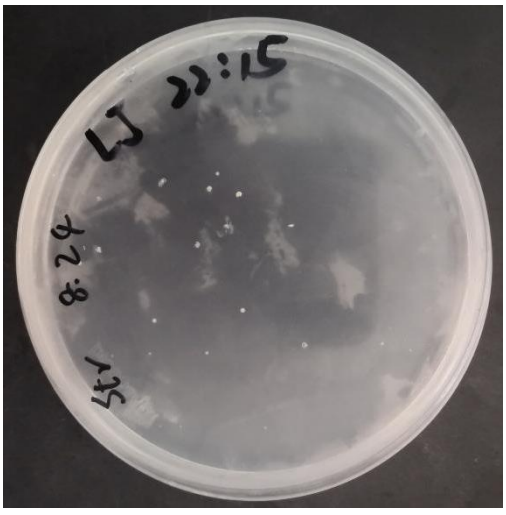


Figure 30 plate growth result (T1 receptive state)

| Colony pcr reaction system (10μL) | |
|---|---|
| *Hmas*-F-2/*aroG*-R-2 | 0.4μL/0.4μL |
| 2×Rapid Taq Master Mix | 5μL |
| template | 1μL |
| ddw | 3.2μL |

| Reaction procedure | | |
|---|---|---|
| 95℃ | 3min | |
| 95℃ | 15s | ×30 |
| 60℃ | 15s | |
| 72℃ | 45s | |
| 72℃ | 5min | |

Figure 31 colony PCR system and procedure

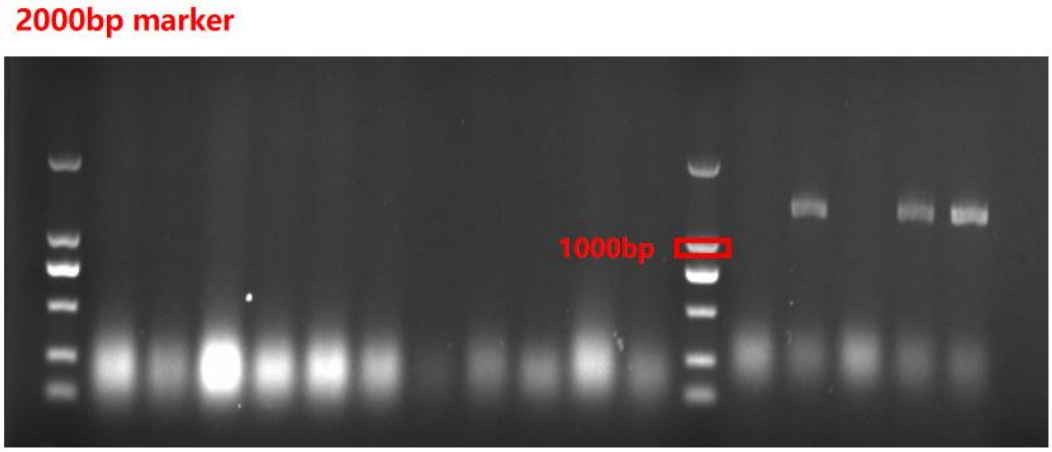



Figure 32 colony PCR result

The bands of strain 13, 14 and 15 were 1200 bp, and the results were correct.

| Reaction system (25μL) | |
|---|---|
| PLB1s-hmas-aroG$^{fbr2}$-phe$^{fbr2}$ | 15uL |
| SalI | 0.5μL |
| rCutsmart | 2.5μL |
| ddw | 7μL |

| Reaction procedure | |
|---|---|
| 37℃ | 2h |

Figure 33 enzyme digestion system and procedure

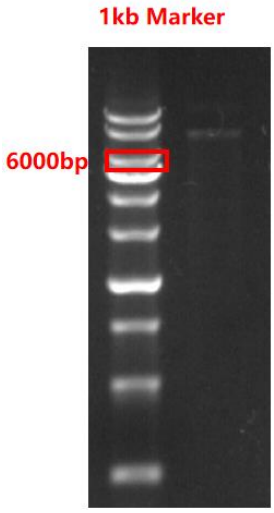



Figure 34 enzyme digestion verification result

The size of 7431 bp band was correct.

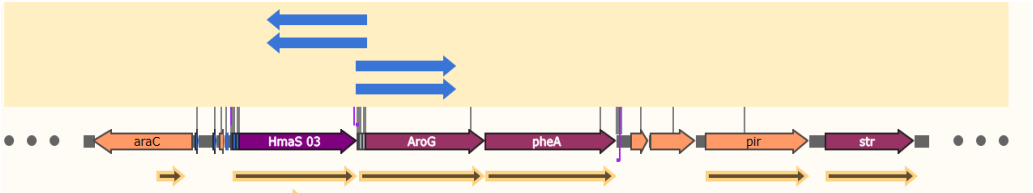



Figure 35 pLB1s-HmaS-aroG$^{fbr2}$-pheA$^{fbr2}$ sequencing result

The sequencing result was correct.

## 9.14 Jiaqi Zhang

### pLB1s-hmaS constructing experiment

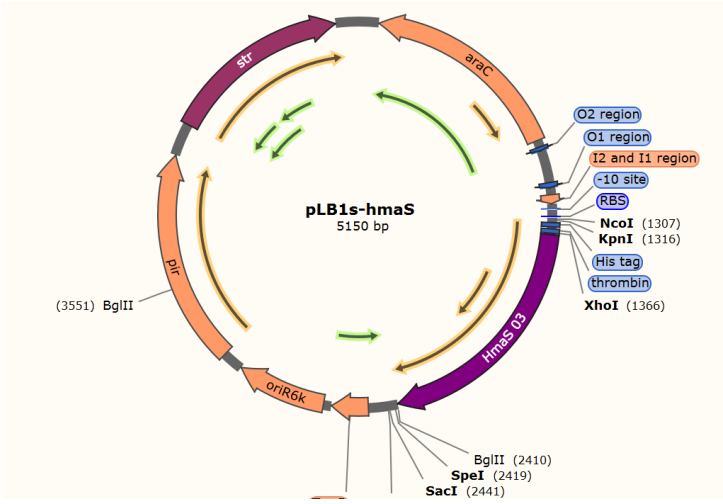



Figure 36 pLB1s-HmaS plasmid map

| Reaction system (50μL) | |
|---|---|
| *ori-Gibson*-F/R | 1μL/1μL |
| *PLB1s-hmas-tktA-ppsA* | 1μL |
| 2×HF Mix | 25μL |
| ddw | 20μL |

| Reaction procedure | | |
|---|---|---|
| 98℃ | 3min | |
| 98℃ | 30s | ×25 |
| 55℃ | 30s | |
| 72℃ | 50s | |
| 72℃ | 10min | |

Figure 37 ORI fragment preparation PCR system (×2)

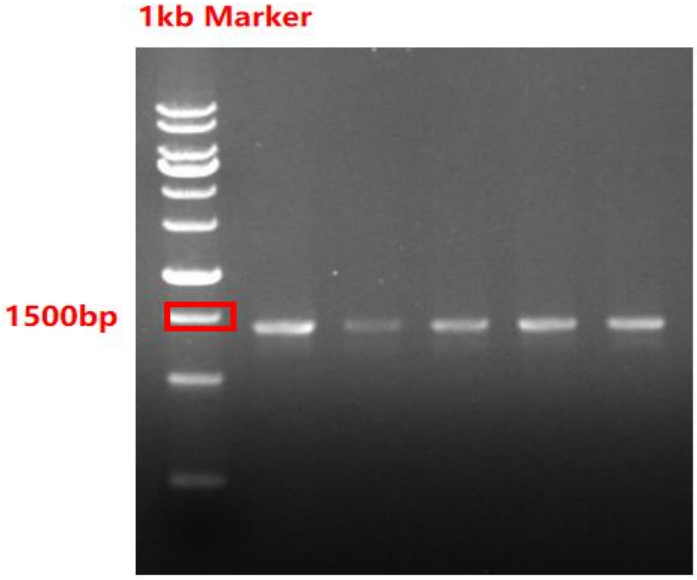



Figure 38 Electrophoresis results

The 1430 bp band of the fragment was correct and there was no heterozygote. The product was purified (two-in-one) at the concentration of 219 ng/uL.

| Reaction system (50μL) | |
|---|---|
| *PLB1s-Gibson*-F/R | 1μL/1μL |
| *PRB1s-hmas* | 1μL |
| 2×HF Mix | 25μL |
| ddw | 20μL |

| Reaction procedure | | |
|---|---|---|
| 98℃ | 3min | |
| 98℃ | 30s | ×25 |
| 60℃ | 30s | |
| 72℃ | 2min | |
| 72℃ | 10min | |

Figure 39 pLB1s-HmaS carrier preparation PCR system (×2)

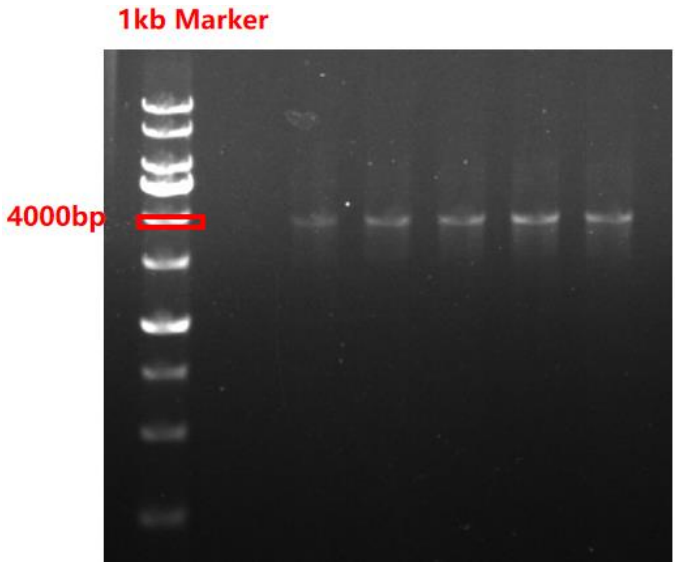



Figure 40 Electrophoresis results

The carrier band was 3700 bp and the product was purified (two-in-one) at a concentration of 223 ng/ul.

| Reaction system (20μL) | |
|---|---|
| PLB1s-hmas | 2.5uL |
| ORI | 1.8μL |
| 5×ceⅡ Buffer | 4μL |
| ExnaseⅡ | 2uL |
| ddw | 9.7μL |

| Reaction procedure | |
|---|---|
| 37℃ | 30min |

Figure 41 Gibson connection system

| Colony pcr reaction system (10μL) | |
|---|---|
| *Hmas*-F-2/*ori*-R | 0.4μL/0.4μL |
| 2×Rapid Taq Master Mix | 5μL |
| template | 1μL |
| ddw | 3.2μL |

| Reaction procedure | | |
|---|---|---|
| 95℃ | 3min | |
| 95℃ | 15s | ×30 |
| 60℃ | 15s | |
| 72℃ | 30s | |
| 72℃ | 5min | |

Figure 42 colony PCR system

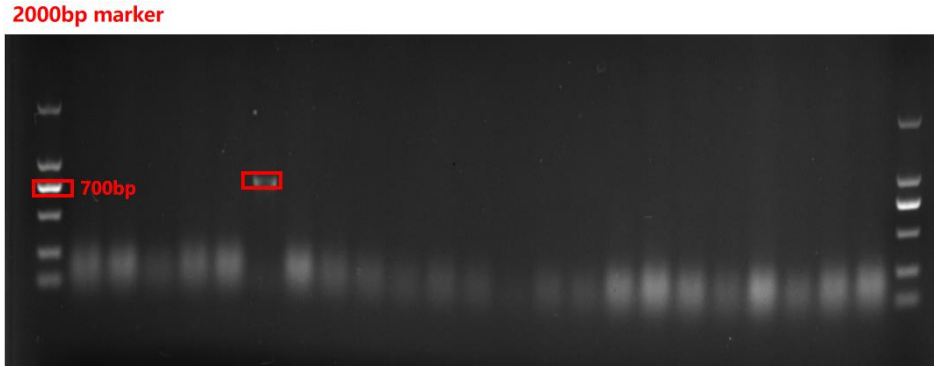



Figure 43 colony PCR result

Correct band is 756 bp, NO.6 is correct.

| Reaction system (25μL) | |
|---|---|
| PLB1s-hmas-aroG$^{fbr2}$-phe$^{fbr2}$ | 15uL |
| SalI | 0.5μL |
| rCutsmart | 2.5μL |
| ddw | 7μL |

| Reaction procedure | |
|---|---|
| 37℃ | 2h |

Figure 44 enzyme digestion system

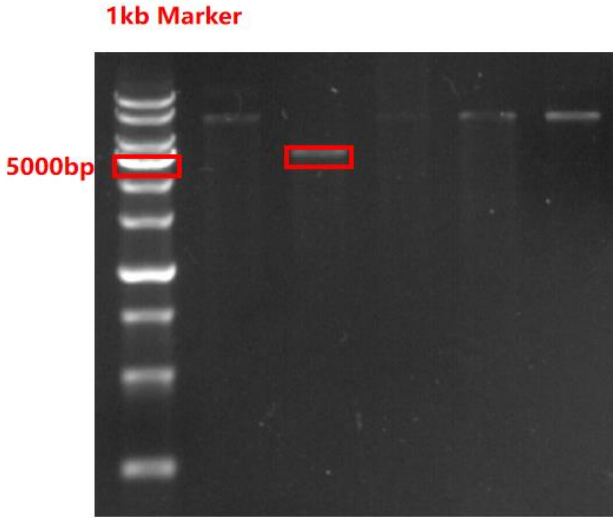



Figure 45 Electrophoresis results

The 5150 bp band was verified by enzyme digestion and the result was correct.