# Week1 (4.1-4.7): construction and functional validation of TPR-CD plasmid

## (A) TPR-CD plasmid construction:

### 1. Amplification of vectors by PCR

Target vector fragments were obtained by PCR with the following RCR system:

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

| PCR | | |
|---|---|---|
| 98° C | 5min | |
| 98° C | 30s | ×25 |
| 58° C | 30s | |
| 72° C | 105s | |
| 72° C | 5min | |
| 25° C | ∞ | |

The polymerase chain reaction product was detected by agarose gel electrophoresis and the correct length of the target fragment was obtained as 3494 bp. we got the correct target fragment and the sample was cut and recovered.

### 2. Amplification of CDase fragments by PCR

The target fragments were obtained by PCR.The RCR system was as follows:

| PCR system (50μL) | |
|---|---|
| 2×Mix | 25ul |
| CD-F | 2ul |
| CD-R | 2ul |
| template | 50ng |
| DDW | 20ul |

| PCR | | |
|---|---|---|
| 98° C | 5min | |
| 98° C | 30s | ×25 |
| 56° C | 30s | |
| 72° C | 42s | |
| 72° C | 5min | |
| 25° C | ∞ | |

The polymerase chain reaction product was detected by agarose gel electrophoresis and the correct length of the target fragment was obtained as 1325 bp. we got the correct target fragment and the sample was cut and recovered.

### 3.Cutting glue recycling

Recover the correct strip by cutting the glue.

## 4. Connecting two fragments using Gibsion assembly method

TPR-CD plasmids were obtained using Gibson (C116) ligation, TPR-V 3494bp, CD 1325bp.

| Gibson system | |
|---|---|
| 0.02 x 3494bp | ng |
| 0.04 x 1325bp | ng |
| 2×c116 Mix | 5ul |
| DDW | to10ul |

| Gibson. | |
|---|---|
| 50° C | 15min |
| 4° C | ∞ |

## 5. Chemical transformation

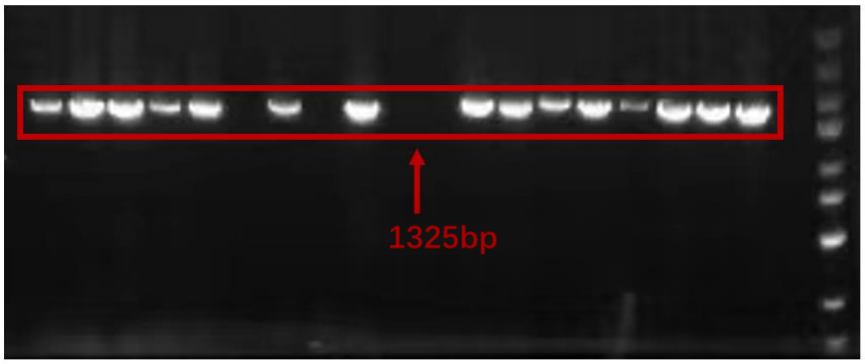
Using the chemotaxis method, the ligation product was transformed into DH5α receptor cells and then spread on LB plates containing Amp resistance and incubated overnight at 37°C in inverted mode.

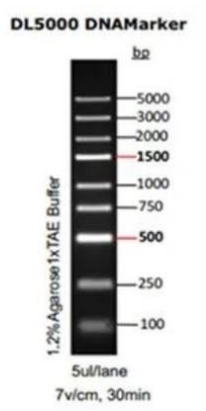
## 6. Colony PCR

After 12 hours of incubation at 37° C, 20 colonies were selected on the plate and the colony PCR system was as follows:

| PCR system (10μL) | | PCR | | |
|---|---|---|---|---|
| 2×Hieff | 25ul | 98° C | 5min | |
| CD-F | 2ul | 98° C | 30s | ×25 |
| CD-R | 2ul | 57° C | 30s | |
| DDW | 4.2ul | 72° C | 50s | |
| | | 72° C | 5min | |
| | | 25° C | ∞ | |

The PCR products were examined by agarose gel electrophoresis with the following results:



### 7. Inoculation with monoclonal

Each of the four monoclones was selected and inoculated into liquid medium supplemented with 1% ampicillin and incubated at 37°C for 12 hours.

### 8. Extract plasmid, send for sequencing

The plasmid was extracted and sequenced, and the TPR-CD sequencing result was correct.

## (B) TPR-CD function validation

### 1. Configuration of M9 solid medium supplemented with different concentrations of 5-FC

### 2. Transformation

5-FC: 30mg/L

5-FC: 60 mg/L

(both with high colony growth)

The plasmid TPR-CD was transfected into BW△CD receptor cells by chemotaxis, and coated on M9 medium supplemented with 30mg/L and 60mg/L 5-FC, and we found that both of them grew, and it was presumed that the CD enzyme was not expressed or was weakly expressed, and that the CD enzyme fragment was derived from the previously constructed gY9s-dual T7-Trrnb HmaS(Scpa1)-Bio 177 plasmid, so we learnt that CDase expression needs to be induced by one step in advance after asking our lab teacher;

### 3. Transformation

The TPR-CD plasmid was transferred into BW△*CD* receptor cells, plates were coated, and single clones were picked and plugged into liquid LB with 0.1% ampicillin added and shaken;

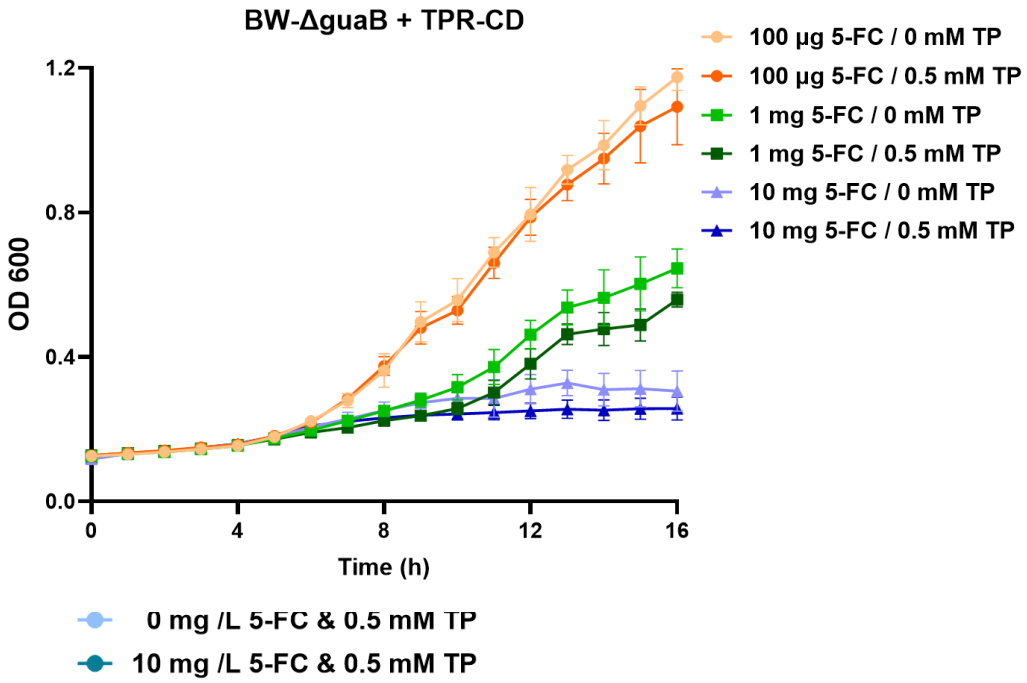
## 4. Induce

The induction system was divided into three groups for doing negative control. Three groups of induction were done with plus ara plus TP, plus ara without TP and without ara with TP for 18h;

| **Induction of system1 (+ara +TP)** | |
|---|---|
| ZY | 4.8mL |
| 50x 5052 | 100 uL |
| 50x M | 100 uL |
| MgSO4 | 10 uL |
| 1000x Trace elements | 10 uL |
| bacterial solution | 50 uL |
| ara | 50 uL |
| A-resist | 5 uL |
| TP (50mM) | 50 uL |
| **Induction of system2 (+ara -TP)** | |
| ZY | 4.8mL |
| 50x 5052 | 100 uL |
| 50x M | 100 uL |
| MgSO4 | 10 uL |
| 1000x Trace elements | 10 uL |
| bacterial solution | 50 uL |

| | |
|---|---|
| ara | 50 uL |
| A-resist | 5 uL |
| **Induction of system3 (-ara -TP)** | |
| ZY | 4.8mL |
| 50x 5052 | 100 uL |
| 50x M | 100 uL |
| MgSO4 | 10 uL |
| 1000x Trace elements | 10 uL |
| bacterial solution | 50 uL |
| A-resist | 5 uL |

## 5. Function verification




The induced bacteria were cultured in M9 test tubes as well as in 96-well plates, and it was found that the intensity of CDase expression was too high with or without the addition of TP, which resulted in the lethality of the strains in 10 mg/L of 5-FC;
The concentration of 5-FC was lowered to make 10 mg/L, 1 mg/L, 100 μg/L, 10 μg/L, 1 μg/L and 0 μg/L, respectively; theophylline concentration was selected as 0 mM TP and 0.5 mM TP, with a total of 6*2=12 groups of conditions, eight parallels in each group, which was done in 96-well plates, and it was found that the addition of TP could turn on the expression of downstream genes but the change was not significant compared with that of the no TP group big, presumably the CD enzyme expression is too strong.

On this basis, we designed to add the rare codon AGGAGA in front of the corresponding sequence of the CDase gene to reduce its expression, so we constructed TPR-CD1 and TPR-CD2 plasmids with one set of one and two sets of rare codons added, which were used to carry out the next experiments.

## Week2 (4.8-4.14): Construction and functional validation of TPR-CD1 and TPR-CD2 plasmids

### (A) Construction of TPR-CD1 and TPR-CD2 plasmids:

The rare codon AGGAGA was added before the corresponding sequence of the CDase gene to reduce its expression, so the TPR-CD1 and TPR-CD2 plasmids with one and two sets of rare codons were constructed and used for the next experiments.

## 1. Amplification by PCR

Using TPR-CD as a template, the rare codon sequences were added to the plasmid sequences in the form of primers, the annealing temperature was changed, and the remaining operations were the same as in the first week's experiments

## 2. Indigest

Reduction of false positives caused by the original template

System: PCR fragment - 1ug-2ug
rCutsmart--5uL
DpnI-1uL
ddw--to 50uL
Procedure: 37°C 2h
80°C 20min
25°C ∞

## 3. Transformation

The digested system was transferred into DH5α receptor cells, coated plates, and incubated in inverted culture at 37°C for 12h;

## 4. Inoculation and plasmid extraction

The operation is the same as that of the previous plasmid.

## 5. Send sequencing

The plasmid was sequenced and the TPR-CD1/TPR-CD2 sequencing results were correct.

## (B) Functional validation of TPR-CD1/2

## 1. Transformation

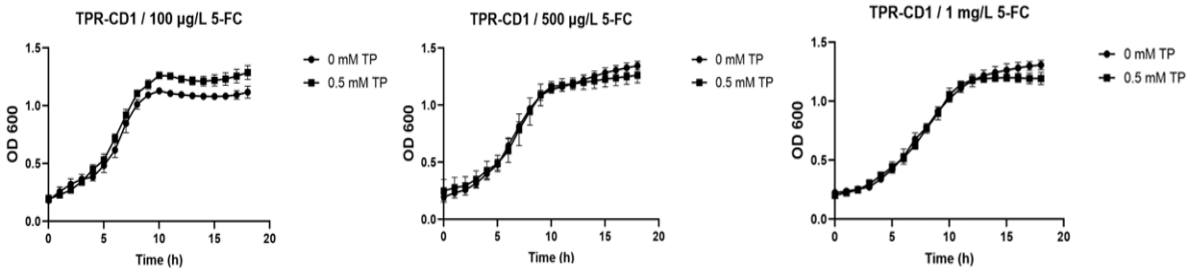
The successfully sequenced TPR-CD1 and TPR-CD2 were transfected into BW△CD receptor cells, coated with plates, and the single clones were picked and accessed into liquid LB with 0.1% ampicillin and shaken;

## 2. Induction

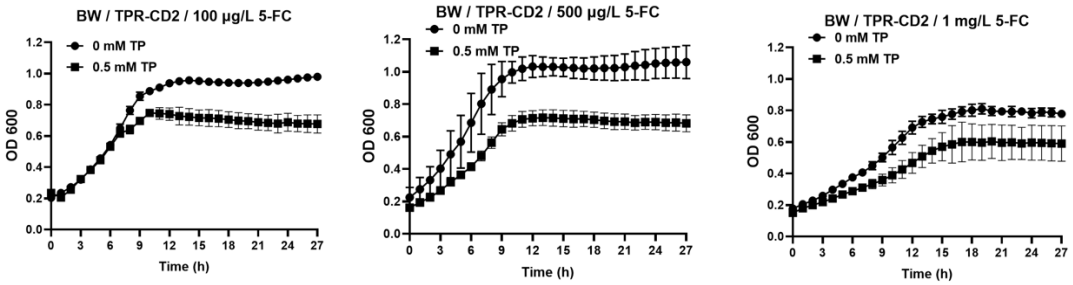
Induction system and operation are the same as before

## 3. Function verification

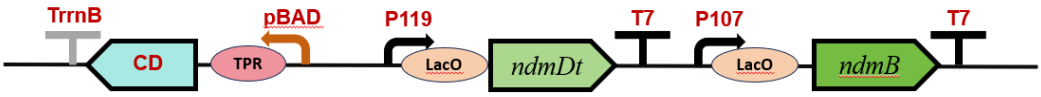
After 18 h of induction, a 5-FC gradient was set up between 100ug/L and 10mg/L, and the




enzyme marker was set overnight;




It was found that TPR-CD with the addition of a 2X rare codon showed better specificity for theophylline, and the addition of theophylline turned on the expression of downstream CD enzymes, which can be used for screening as of now, so we planned to construct the TPR-CD2-DtB plasmid, with ndmDt initiated by P119, ndmB initiated by P107, and TPR-CD initiated by pBAD, to couple the ability of theophylline degradation with the growth of the strain Coupling.




## Week3 (4.15-4.21): Construction and function validation of TPR-CD2-DtB plasmid

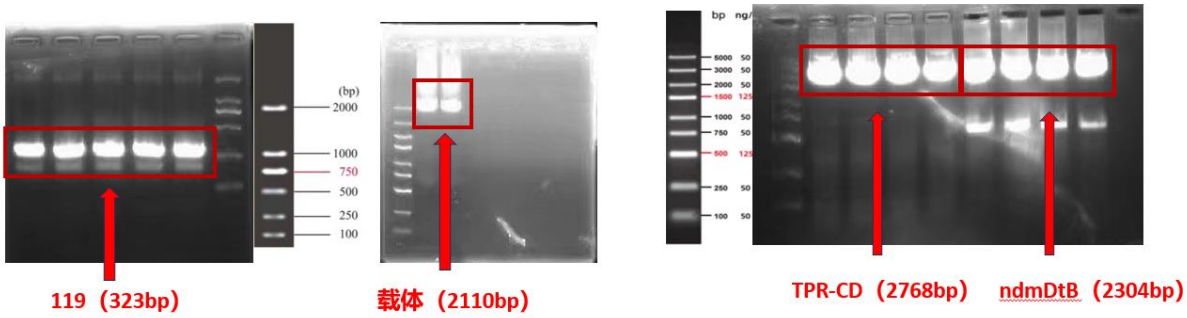
### Initiation of ndmDt by P119, ndmB by P107 and TPR-CD by pBAD coupled the ability to degrade theophylline to strain growth

### (A) Construction of TPR-CD2-DtB:

### 1.PCR + cut gel recovery:

| extract (from book etc) | primer | lengths | annealing temperature | Extended time |
|---|---|---|---|---|

| | | | | |
|---|---|---|---|---|
| 119 | 119-F/R | 323bp | 53° C | 10s |
| TPR-CD | TPR-CD-F/R | 2768bp | 59° C | 84s |
| ndmDtB | DtB-F/R | 2304bp | 56° C | 69s |
| vector (epidemiology) | DtB-V-F/R | 2110bp | 59° C | 66s |




## 2. Connection

The four fragment golden gate connection is made in the same way as in the first week.

## 3. Transformation

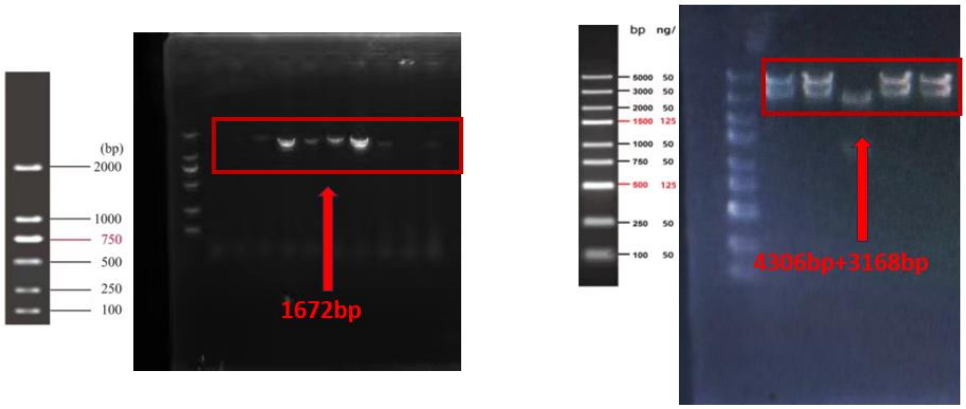
The ligated system was transferred into DH5α receptor cells, coated with plates, and cultured inverted at 37°C for 12h;

## 4. Colony PCR

PBF/PBR was chosen as the primer with a theoretical length of 1672bp;

## 5. Plasmid extraction + digestion verification

EcoRI was selected for double digestion with HindIII with 80% positivity




## 6. Send sequencing

The correctly digested protoplasmid was sent for testing, and TPR-CD2-DtB was sequenced correctly.

## (B) Function validation of TPR-CD2-DtB:

### 1. Transformation

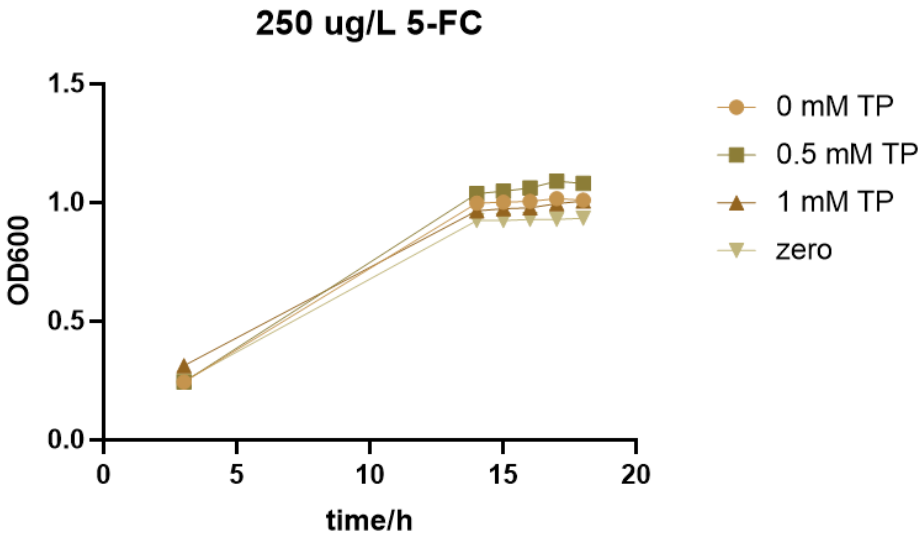
The successfully sequenced TPR-CD1 and TPR-CD2 were transfected into BW△*CD* receptor cells, coated with plates, and the single clones were picked and accessed into liquid LB with 0.1% ampicillin added and shaken;

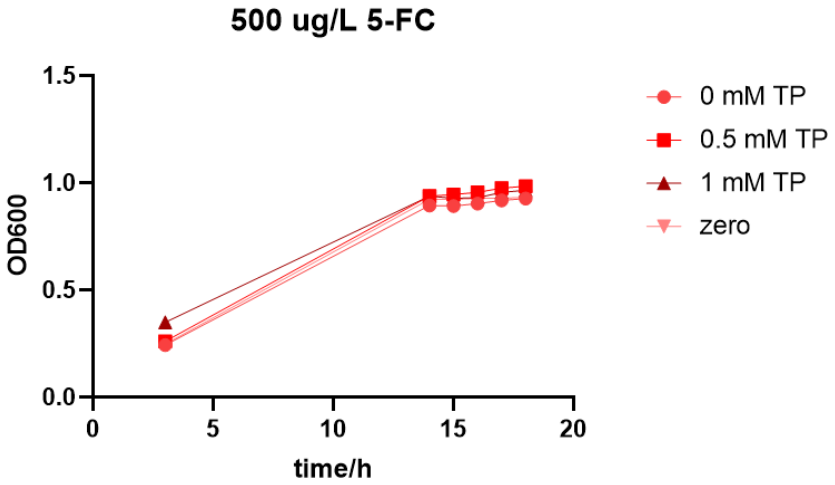
### 2. Induction

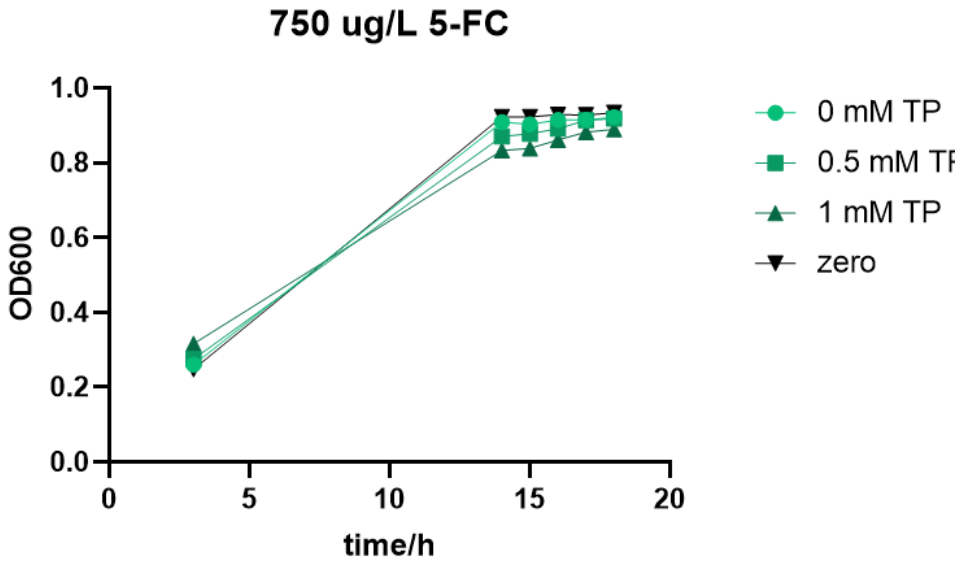
| Induction of system4 | |
|---|---|
| ZY | 4.8mL |
| 50x 5052 | 100 uL |
| 50x M | 100 uL |
| MgSO4 | 10 uL |
| 1000x Trace elements | 10 uL |
| bacterial solution | 50 uL |
| ara | 50 uL |
| A-resist | 5 uL |

### 3. Function verification

Configure the M9 culture solution in advance, set the 5-FC concentration to 0ug/L, 250 ug/L, 500 ug/L, 750 ug/L, and the TP concentration to 0mM, 0.5mM, 1mM according to the pre-experiment of the previous TPR-CD2 function validation, and use 96-well plate to do the parallel experiments, add 2% of the bacterial solution (200uL) to each well, and measure the growth in the zymography overnight at 30°C Curve;

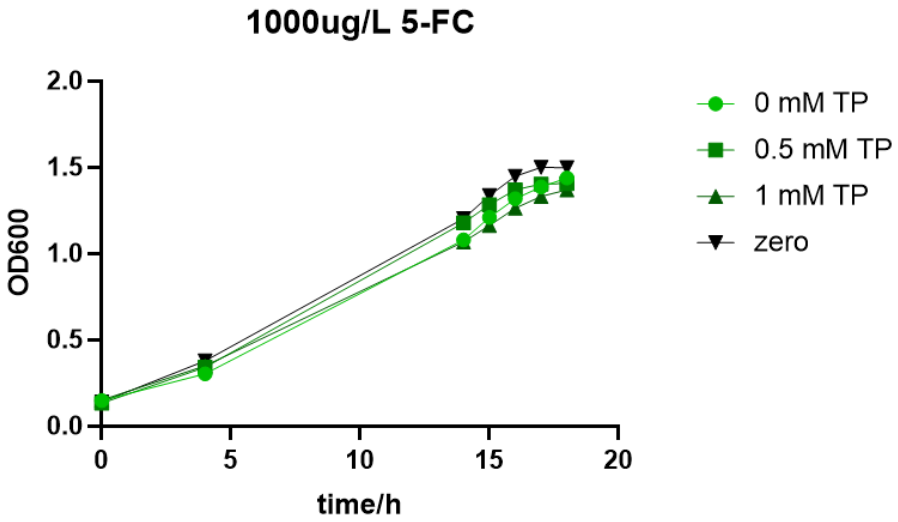

250 ug/L 5-FC
OD600
1.5
1.0
0.5
0.0
0
5
10
15
20
time/h
0 mM TP
0.5 mM TP
1 mM TP
zero

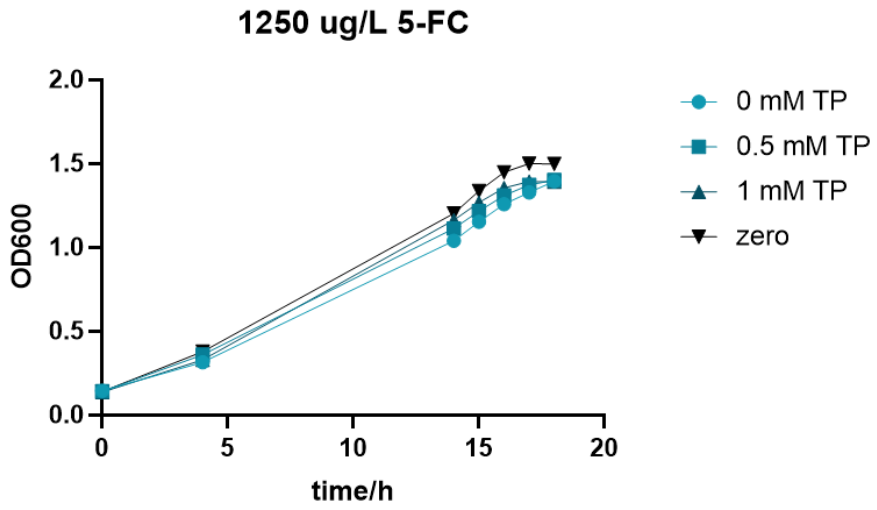

500 ug/L 5-FC
OD600
1.5
1.0
0.5
0.0
0
5
10
15
20
time/h
0 mM TP
0.5 mM TP
1 mM TP
zero

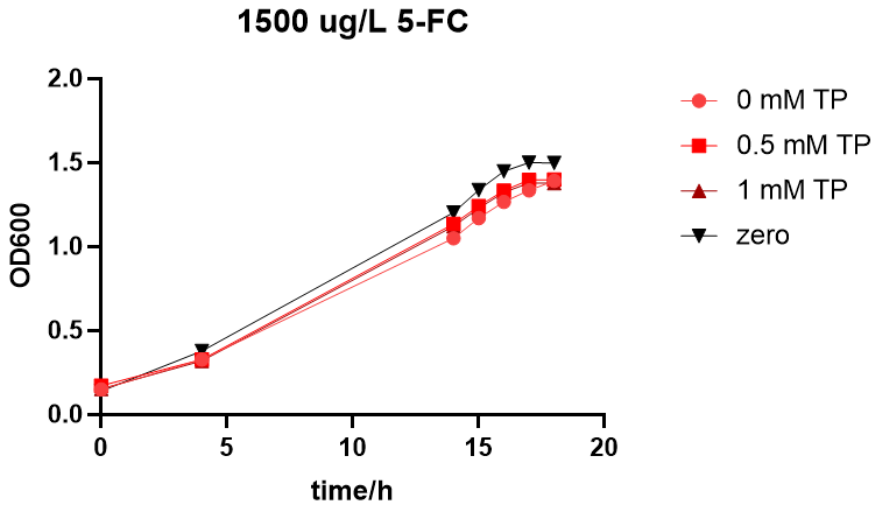



## 4. Further validation

From the plotted growth curves we found that TPR-CD2-DtB was not sensitive to low doses of 5-FC, so we planned to increase the concentration of 5-FC to further verify the function of TPR-CD2-DtB, and we set the concentration of 5-FC to 1000ug /L, 1250ug/L, 1500 ug/L, and the concentration of TP was still 0mM, 0.5mM , 1 mM, and the same operation was performed to plot the second growth curve;






We found that the survival rate of the TPR-CD2-DtB strain did not significantly decrease with increasing concentrations of 5-FC compared to the blank control, and we speculated that the large size of the plasmid might have reduced the ability of TPR to bind theophylline and turn on the downstream CD enzymes or led to the restricted expression of TPR, which also suggests that TPR-CD2-DtB is not sufficient to be used as a screen for the degradation of the TP-producing 1-MX strains, and ultimately we decided not to use directed evolution of TPR-CD2-DtB to obtain a specific sensor for PX.

## Week4 (4.22-4.28): guaB Knockout

## (A) Preparation of targeting fragments

### 1.PCR to obtain target fragments

Primers were designed, E. coli *BW25113* original strain was used as a template PCR to obtain guaB-up and guaB-down fragments; pccdK2-up-kan-down was used as a template PCR to obtain kan fragment; pccdK2-O was digested with Kpn1 to obtain the vector fragment pccdK; the operations were all the same as in the first week, with the replacement of the primers, the annealing temperature and the extension time.

### 2. Connections

The three fragments were joined with Gibson to obtain the pccdK-up-kan-down hit fragment.

## (B) Preparation of electrotransferred sensory states

### 1. Water-washed organisms

200 μL of bacterial solution was removed from the preserved glycerol tube and added to 5 mL of LB liquid medium. After incubation at 30°C and 200 rpm for about 12 h, the culture was transferred to a new LB medium at 2% inoculum.

### 2. Induction

After 0.5 h of incubation, add arabinose at a final concentration of 0.2%; incubate at 30°C for about 2 to 2.5 h until the OD600 reaches 0.55-0.6 (either too high or too low will severely affect the efficiency of the electrotransferred sensory state). Remove the culture solution and let it stand on ice for 30 minutes. Pre-cool the centrifuge rotor to -80°C and let stand for 10 minutes.

### 3. Centrifugation

Collect the organisms in pre-cooled centrifuge tubes, kept on ice at all times, and centrifuge at 4000 rpm for 10 minutes; dispense 50 mL per tube. gently aspirate and beat with 18 mL/tube of ice-pre-cooled, sterilised 10% glycerol, kept on ice at all times, and dispense into two 10 mL centrifuge tubes. Centrifuge at 4200 rpm for 10 minutes using a cryogenic centrifuge; immediately after completion of centrifugation, pour off the supernatant and gently lance it, keeping it on ice at all times. Repeat the above steps three times, using 5 mL of ice pre-cooled sterilised 10% glycerol for the first two washes. The last time the organisms were resuspended using 0.25 mL of 10% glycerol and the two tubes were combined into one.

### 4. Dispensing

Dispense 100 μL into pre-cooled centrifuge tubes, which can be electrotransformed or immediately stored in a -80°C refrigerator, and this sensory state can be stored at -80°C for half a year.

## (C) Knockouts

Take 100 ul of receptor cells mixed with 200 ng or more of the targeting fragment and incubate on ice for 10-30 min. Wash and dry the electrotransfer cup, and place it in an ultra-clean table for UV sterilisation for 20 minutes. Subsequently, the sensory cells were quickly transferred to the bottom of the electrotransferring cup, the wall of the cup was wiped dry, and the cells were put into the electrotransferring instrument for electrotransferring. After electrotransformation, 1000 μL of 37℃ pre-warmed LB medium was added immediately, gently mixed, and transferred to a 1.5 mL centrifuge tube. After incubation in a shaker at 30°C and 150 rpm for 45-60 min, remove and spread on a selective plate containing Kana and Str double antibodies.