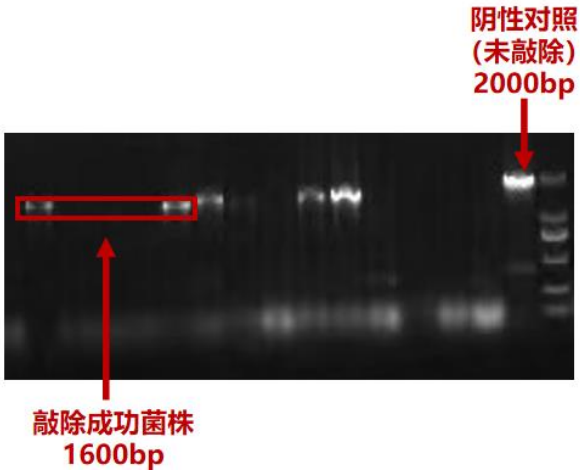
Week1 (5.1-5.7): validation of *guaB* knockout

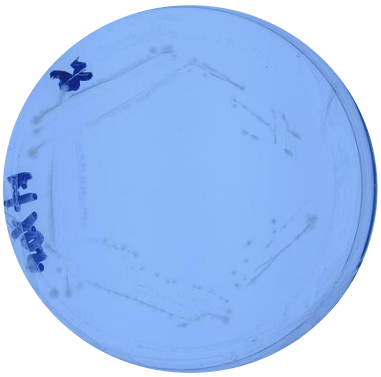
(i) Validation of *guaB* knockout

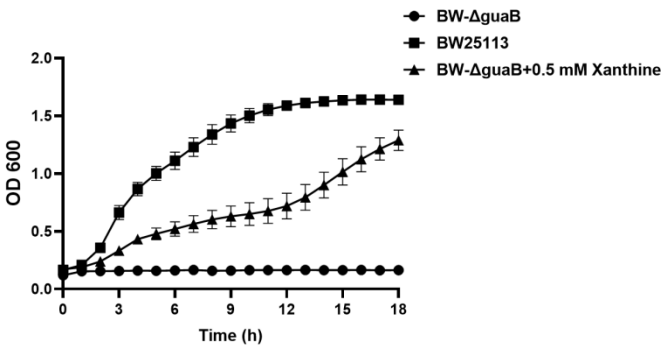
1. Colony PCR



2. Effectiveness Verification

Two monoclones were selected for validation. They were cultured on M9 medium containing 0.2 mM xanthine and M9 medium without xanthine, respectively.After 24 hours of incubation at 37°C, the knockdown effect was verified using plate streaking. The results showed that the strains were able to survive on the xanthine-containing medium, while the strains did not survive on the xanthine-free medium.





Subsequently, we compared the wild-type strain with the knockout strain. This result validated the necessity of xanthine and paraxanthine for bacterial growth, demonstrated that the sensor system can accurately respond to the presence of substrates, and provided a reliable basis for subsequent screening experiments.

3. Further validation

Transform the pYB1s-ndmDCBAE plasmid into the BW-ΔguaB sensory state (this strain is capable of converting caffeine to xanthine). Activate at 37°C until the bacterial solution is turbid.

Induction was performed using the ZYM5052 culture system, using arabinose and IPTG as inducers (to enhance plasmid expression).

After 36 hours of incubation at 25°C on a shaker, the bacteria were inoculated into the following three M9 media for plate streaking: medium without caffeine and xanthine, medium with caffeine and without xanthine, and medium with xanthine and without caffeine. No colony growth was observed on the xanthine-added medium only and the validation was successful.

Week2 (5.8-5.14): Construction of pYB1s-ndmDCEA plasmid

1. Amplification of vectors by PCR

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

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

PCR		
98° C	5min	
98° C	30s	×25
60° C	30s	
72° C	210s	
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 6991 bp. we got the correct target

fragment and the sample was cut and recovered.

2. Amplification of ndmA fragments by PCR

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

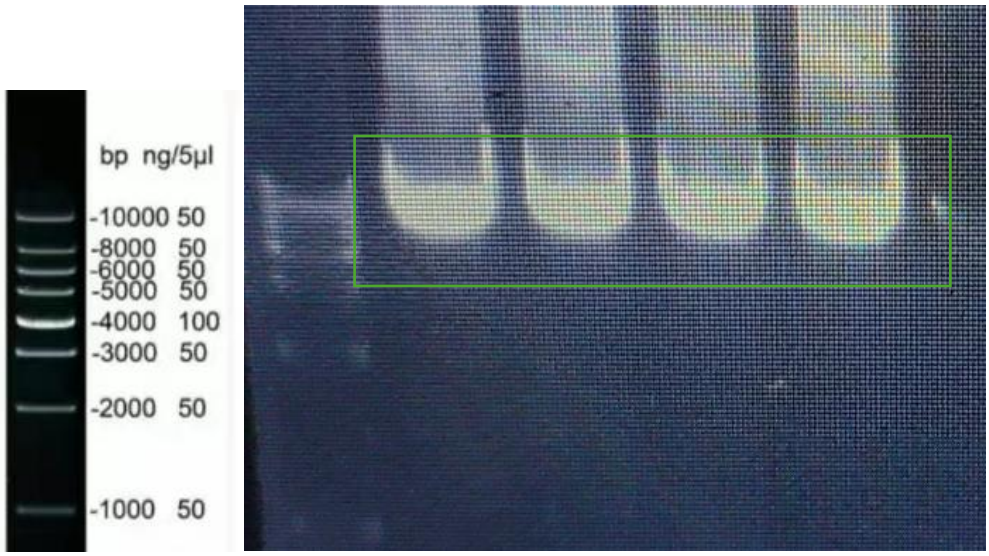
PCR system (50μL)	
2×Mix	25ul
ndmA-F	2ul
ndmA-R	2ul
template	50ng
DDW	20ul

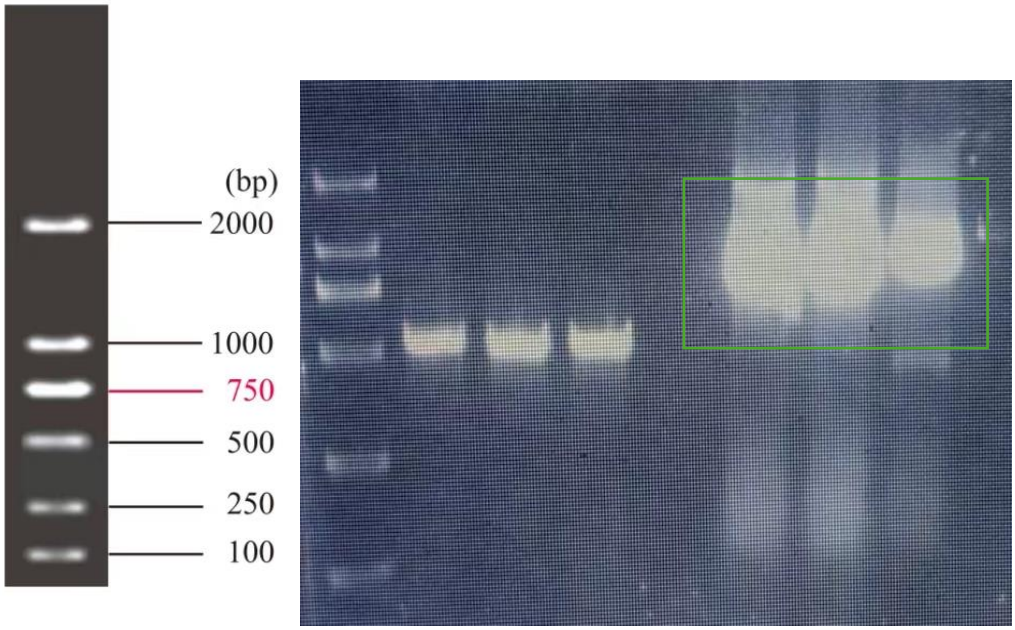
PCR		
98° C	5min	
98° C	30s	×25
57° C	30s	
72° C	35s	
72° C	5min	
25° C	∞	

The polymerase chain reaction product was detected by agarose gel electrophoresis and the correct length of the target fragment was obtained as 1115 bp. we got the correct target fragment.

3.Cutting glue recycling

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





3. Gibsion Connection

The pYB1s-ndmDCEA plasmid was obtained using Gibson (C116) ligation with vector DCE 6991bp and ndmA 1115bp.

Gibson system	
0.02 x 6991bp	ng
0.04 x 1115bp	ng
2×c115 Mix	5ul
DDW	to10ul

Gibson.	
50° C	15min
4° C	∞

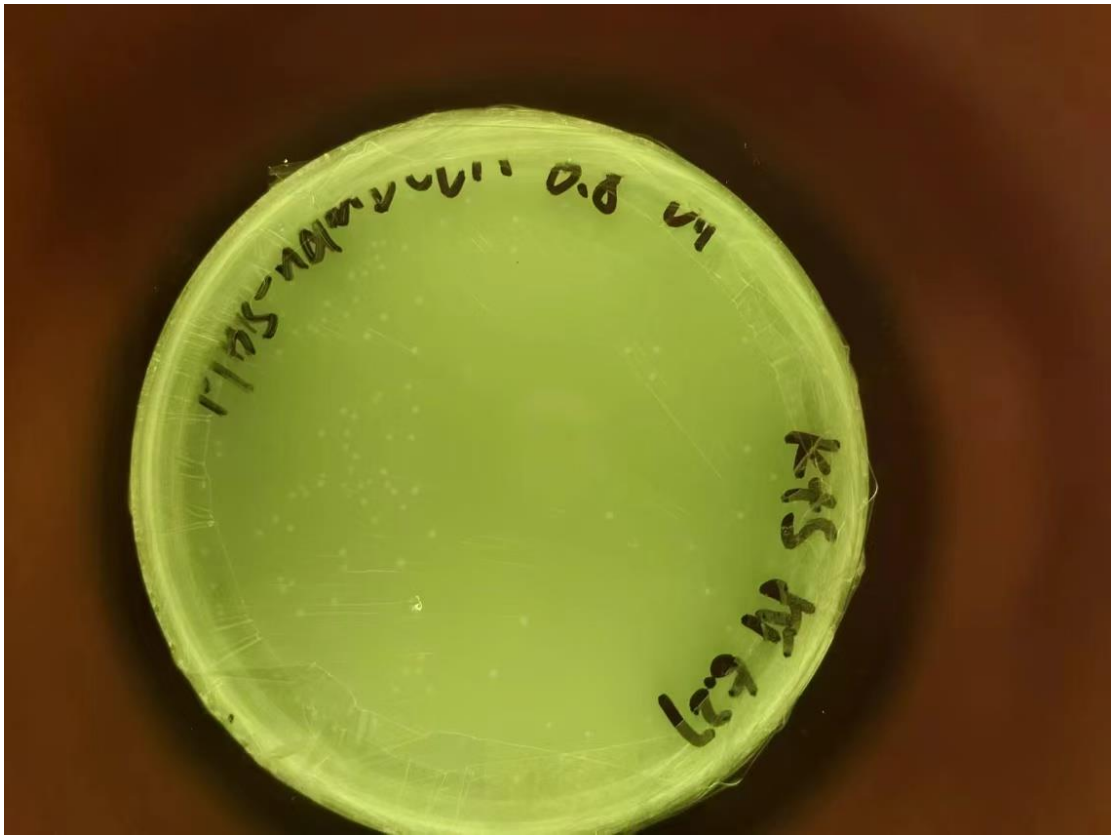
5. Chemical transformation

Using the chemical transformation method, the constructed pYB1s-ndmDCEA plasmid was transfected into DH5α and then coated onto LB plates containing chloramphenicol and incubated at 37°C overnight.

6. Monoclonal inoculation

Colonies were grown on plates after overnight incubation at 37°C. The monoclones were inoculated into liquid medium and incubated at 37°C for 12 hours.

Expected resistant single colonies were grown on the plates and single colonies were subsequently selected for colony PCR.

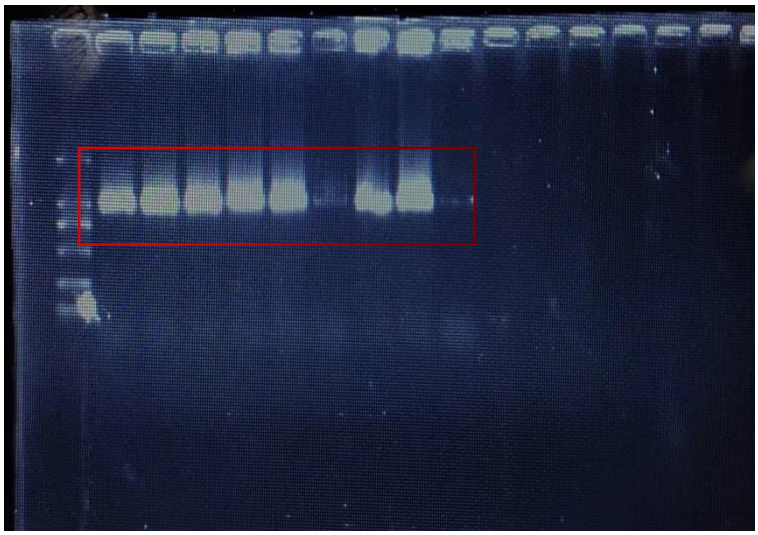


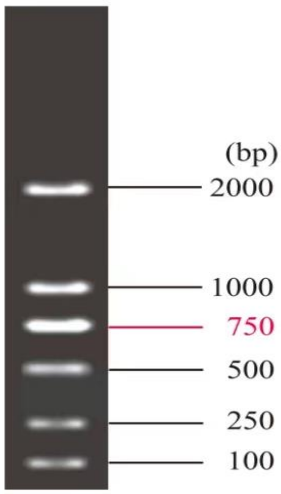
7. Colony PCR

After 12 hours of incubation at 37°C, 10 colonies were selected on the plate and the colony PCR system was as follows:

PCR system (10μL)	
2×Taq enzyme	5ul
ndmA-F	0.4ul
ndmA-R	0.4ul
DDW	4.2ul

PCR		
98° C	5min	
98° C	30s	×25
57° C	30s	
72° C	35s	
72° C	5min	
25° C	∞	





The PCR products were examined by agarose gel electrophoresis with the following results: The target band was 1100 bp with 78% positivity.

8. Inoculation with monoclonal

Four monoclones were selected and inoculated into liquid medium and incubated at 37°C for 12 hours.

9. Plasmid extraction + digestion verification

Double digestion was verified using KpnI and HilndII-IHF, and the digestion products were detected by agarose gel electrophoresis. The correctly digested protoplasmid was sent for sequencing.

10. pYB1s-ndmDCEA sequencing result is correct.

Week3 (5.15-5.21): Function validation of pYB1s-ndmDCEA

In order to provide a reliable sensor for subsequent experiments, we verified its function. Firstly, we tested the BW25113 strain, which was knocked out of the xanthine synthesis gene, by adding different substrates and observed its growth.

1. Transformation

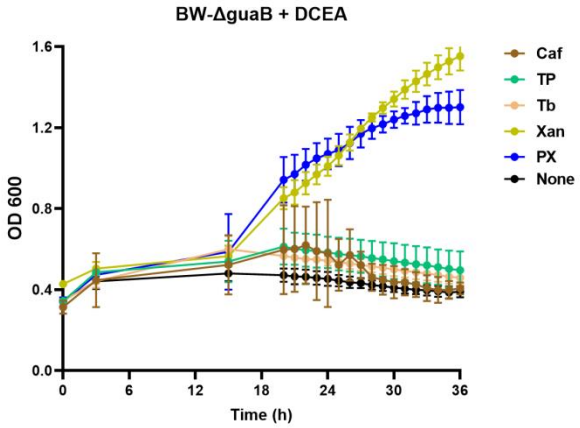
The successfully constructed pYB1s-ndmDCEA plasmid was transferred into the *BW△guaB* receptor state, picked single clones, inoculated and used for the next step of functional verification.

2. Function verification

Configure M9 medium (without xanthine), add caffeine, theophylline, theobromine, xanthine and paraxanthine to different M9 tubes, and set up parallel and blank controls, and incubate at 25°C.

After 18h, samples were taken into 96-well plates and incubation was continued for 18h in an enzyme labeller to plot the growth curve.

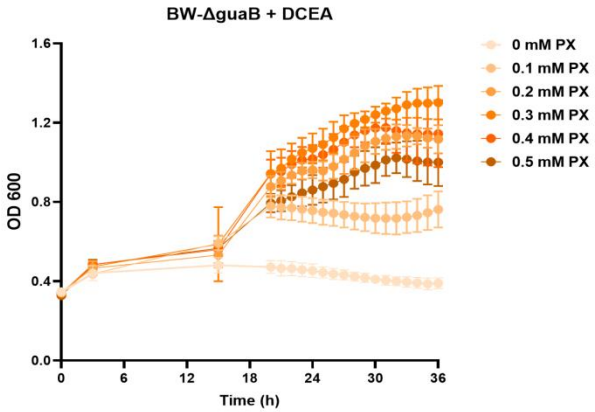
In order to provide a reliable sensor for subsequent experiments, we verified its function. Firstly, we tested the BW25113 strain, which was knocked out of the xanthine synthesis gene, by adding different substrates and observed its growth.



We found that the strain knocking out the xanthine synthesis gene survived only in the presence of xanthine and paraxanthine. This proves the necessity of xanthine for the growth of the strain and also proves the effectiveness of the sensor change by demonstrating that the plasmid is able to degrade paraxanthine into xanthine.

3. Further validation

In order to further verify the tolerance of the sensor strain to PX, we tested the BW25113 strain, which knocked out the xanthine synthesis gene, with the addition of different concentrations of paraxanthine, and plotted the growth curves to observe its growth.



The results showed that DCEA embodied a better gradient in the PX solution from 0 to 0.3 mM, but the growth of the strain was inversely effective as the PX concentration became higher. We speculate that maybe PX also inhibits the growth of bacteria like caffeine.

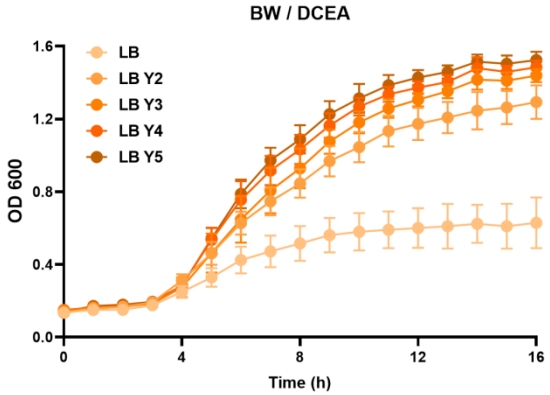
Week 4 (5.22-5.28): Summarise the previous experiments and design the next experimental programme.

(i)Summary of the previous phase of the experiment

In the course of the above experiments, we first induced the *BWΔguaB* strain transfected with pYB1s-ndmDCEA, and a small amount of xanthine powder was added at the time of induction. After the induction was completed the bacterium was washed with water, resuspended with M9 and 10% inoculated into M9 containing methylxanthine. However, it took a long time of incubation (36h) to see the difference, and the bacteria all started to be inhibited or died only after the first growth, so we guess it might be related to the xanthine added at the time of induction was not completely dissolved.

(ii) New experimental designs

In order to make the bacteria grow faster and survive longer, we tried to add different amounts of yeast extract in LB and plotted the growth curve by enzyme marker.



It was found that LB medium with twice the yeast extract was already able to grow the bacteria very well, so we decided to use LB medium with twice the yeast extract for the induction of pYB1s-ndmDCEA in subsequent experiments.

Next, we will perform targeted-site saturation mutagenesis and screening.