February

Project brainstorming, team member selection.

March、April

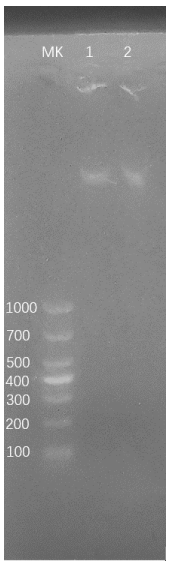
Project selection, communication and conversation with teachers to refine and change our project.
All team members undergo training on lab safety and biosafety.
Members of the lab group, artwork group, web group, and HP group were divided to determine their work.
The lab group was divided into three groups to work on our project.

May 1st-5th

Team members begin to enter the lab to familiarize themselves with the environment.
Configure LB solids and culture medium for backup.

May 10th

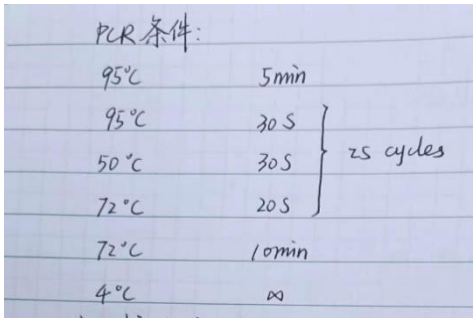
Bacillus subtilis genome extraction, using Zomanbio Genome extraction kit for Gram-positive bacteria.

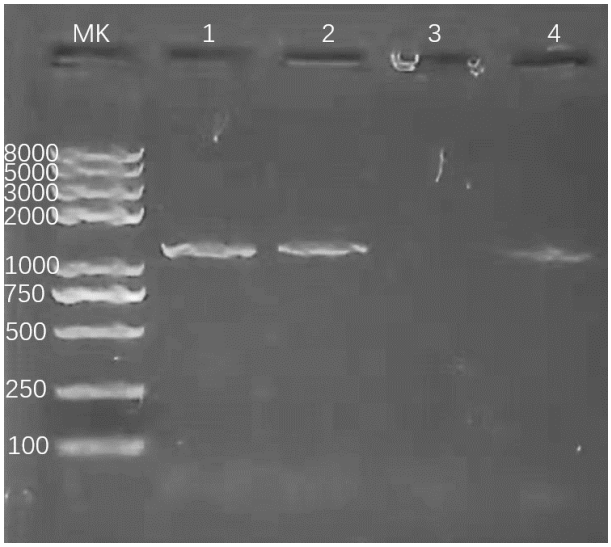


May 12th

capA(BBa_K3796002)Amplification from the *Bacillus subtilis* genome to obtain

ingredient	dosage/ul
template	0.5
dNTPs (10 mM)	0.5
Phanta Super-Fidelity DNA ploymerase	0.5
Phanta Super-Fidelity 2× Buffer	12.5
primer	1+1
ddH20	9





May 13th

capB（BBa_K3796003）amplification from the *Bacillus subtilis* genome to obtain

May 16th

capC（BBa_K3796004）amplification from the *Bacillus subtilis* genome to obtain

May 21st

Genome extraction of *Corynebacterium glutamicum* ATCC 13032

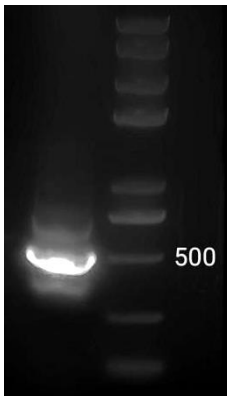
June

Interviews with teachers, PhD's, professors and others to further communicate the progress of the project.
Procure pXMJ19 and pK18mobsacB plasmids.
Contact with company to synthesize promoter and terminator sequences.

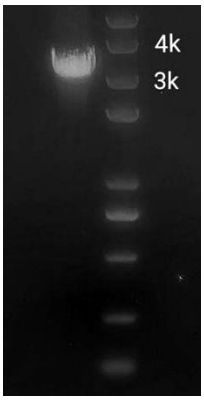
July 5th – July 11th

The target gene MazF was obtained by PCR

Amplification of ppc-UP & ppc-DOWN
ppc-UP & ppc-DOWN were amplified from the genome of *Corynebacterium glutamicum* ATCC 13032 by PCR.



lane1:ppc-UP; lane2:marker



lane1:ppc-DOWN; lane2:marker

July 11th – July 18th

1. Extract plasmid pRJPaph-bjGFP
2. PCR amplification of fluorescent protein gene GFP
3. Plasmid extraction (pk18mobsacB)

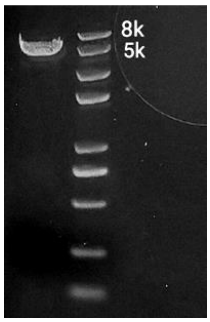
July 19th

Transformation of Escherichia coli DH5 with plasmid pXMJ19 α amplification

Restriction digestion of pk18mobsacB
pk18mobsacB was digested by SmaI (Takara).

10×QuickCut buffer	2μl
Plasmid	10μl
QuickCut SmaI	1μl
ddH_2O	7μl

Incubate at 30℃ for 5 minutes.



lane1:SmaI-pk18mobsacB;lane2:Marker

July 21st

Plasmid Extraction and Amplification of P0864 (BBa_K3796000) and T7 terminator (K2598024) from Synthetic Samples from Biologics

July 23rd

Amplify capA using primers and make it upstream plus RBS sequence and both upstream and downstream plus homology arm for seamless cloning in the next step.No product was detected the first time due to power failure of the device.

July 24th

Amplify capC with primers and add RBS sequence upstream and homologous arms upstream and downstream for seamless cloning in the next step.

July 25th

Detection of gel recovery products of capA capC PCR

July 26th

1. Plasmid pXMJ19 was extracted and linearized (Sma I)
2. The plasmid pXMJ19-mazF was seamlessly cloned and transformed into E. coli DH5 α

Amplify capB using primers and make it upstream plus RBS sequence with homologous arms both upstream and downstream for seamless cloning in the next step.

Amplify the T7 terminator from the extracted plasmid using primers with the homology arm added both upstream and downstream for the next step of seamless cloning.

T7 PCR product gel recovery electrophoresis assay.

P0864 was amplified from the extracted plasmid with homologous arms upstream and downstream. The bands are not homogeneous, plan to re-PCR

July 27th

CapB gel recovery product electrophoresis

Re-amplify P0864 from the extracted plasmid with homologous arms upstream and downstream. Gel recovery was performed.

P0864 PCR product recovery

July 28th

Prepare chloramphenicol resistance medium.
Extracted plasmid pXMJ19 for assay. Extraction failed.

July 29th
Extracted pXM19. very dark band near 6000 bp, darker band at > 10000 bp, similar to the third group of previously successfully extracted pXMJ19 plasmids; 5 and 6 were re-electrophoresed for 7.28 extracted plasmids, indeed still no visible band.

The concentration of the plasmid solution was determined to be 10.8 ng/uL and 11.4 ng/uL, respectively, which is too low to be used for subsequent digestion.

July 30th

pXMJ19 extracted.
No band.
As some pXMJ19 was presented on 7.29, the concentration was low and an attempt was made to use these plasmids for re-transformation.
(1) pXMJ19 1 uL + DH5α mix, ice bath for 10 min.
(2) Water bath at 42°C for 60 s.
(3) Ice bath for 2 min.
(4) Add 500 uL of unresistant LB and mix well.
(5) Recovery incubation at 37°C for 25 min in a 180 rpm shaker.
(6) Centrifuge at 10,000 rpm for 15 min, retain 100 uL, and coat the plate.
(7) Incubate overnight at 37°C in inverted position.

July 31st

Extraction of pXMJ19 plasmid.
The plasmids were extracted in 8 copies using the bacterial broth obtained from the 7.30 shake. 1234 used the Ritai kit (the missing P3 was replaced by the Novozymes kit) and 5678 used the Novozymes kit.

Translated with www.DeepL.com/Translator (free version)

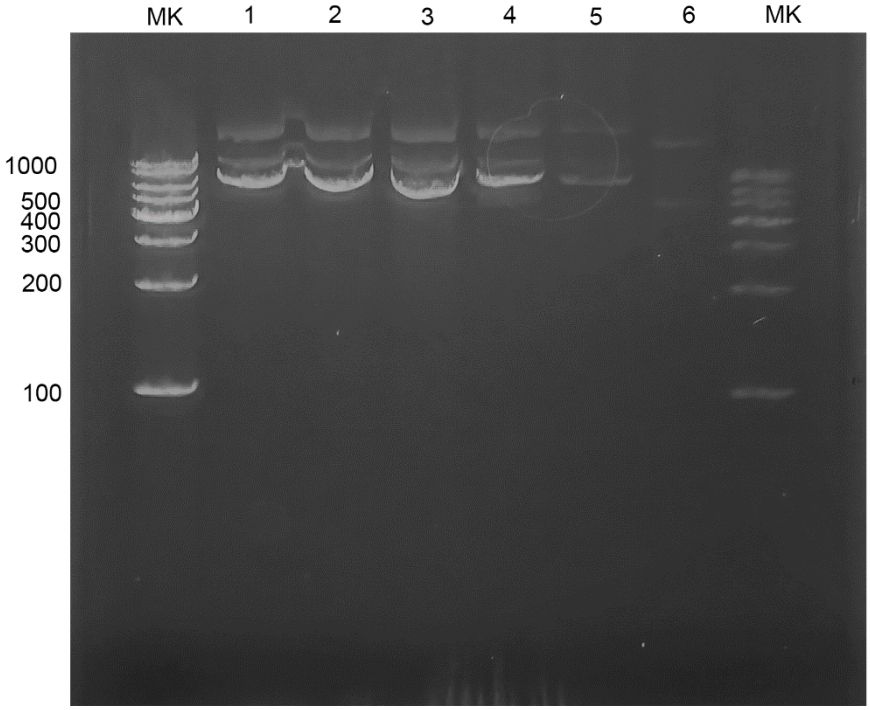
1, 2, 5 and 6 have no bands; 3 and 7 have bands near 6000 bp and > 10000 bp, similar to the third group of previously successfully extracted pXMJ19 plasmids; 4 and 8 are presumed to undergo degradation.

The concentrations were measured separately for 3 and 7. Two measurements were averaged.

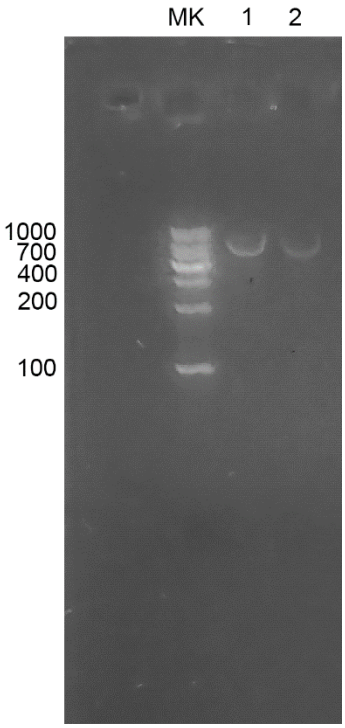
sample	concentration(ng/uL)	A260/A280	A260/A230
3	105.1	2.13	2.29
7	14.3	2.00	3.28

Extract pXMJ19 again

Extraction was successful and XmaI single digestion was performed.



Gel recovery and testing.



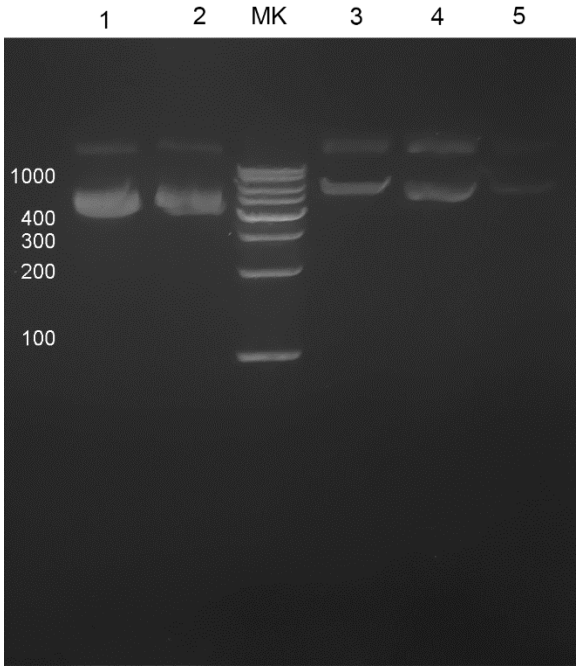
August 1st

The capA module (former part of BBa_K3796008) was cloned seamlessly. Transformation into E. coli DH5a

August 2nd

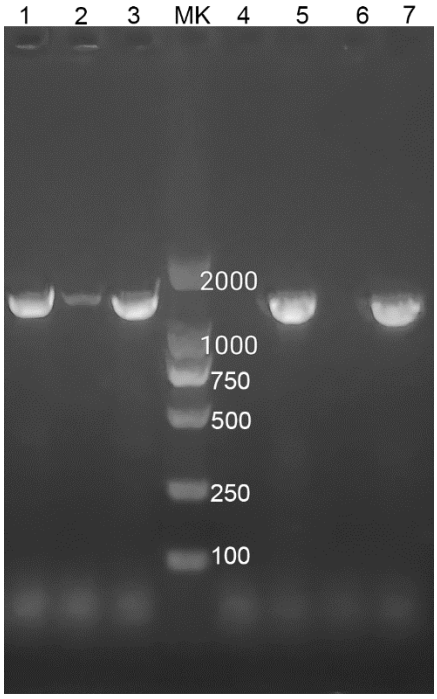
1. PCR was used to obtain the target fragment base promoter Patp2
2. The plasmid pXMJ19-GFP was seamlessly cloned and transformed into E. coli DH5 α
3. Extract plasmid pXMJ19-mazF

Continue extraction of pXMJ19 plasmid.
Xmal enzyme cleavage of pXMJ19

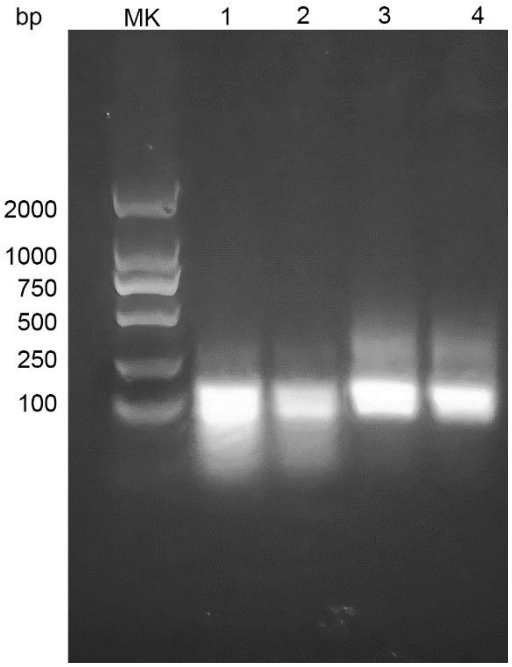


August 3rd

PCR assay after seamless cloning transformation.

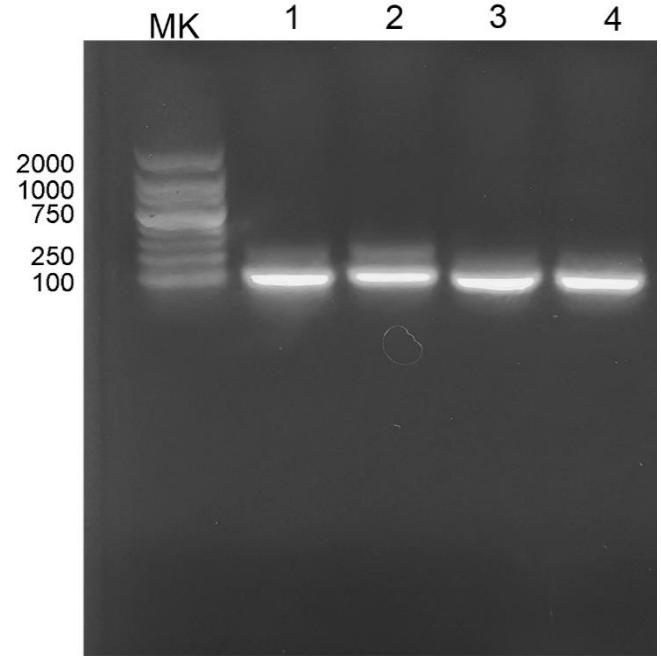


PCR additions for the P0864 T7 homologous arm of the capB module construct were applied.



August 4th

P0864 T7 homology arm PCR addition and gel recovery assay for capC module construction.



P0864 T7 homology arm PCR addition and gel recovery assay for capC module construction.

August 5th

Seamless cloning of transformed Petri dishes for observation. Basically no colonies grew.

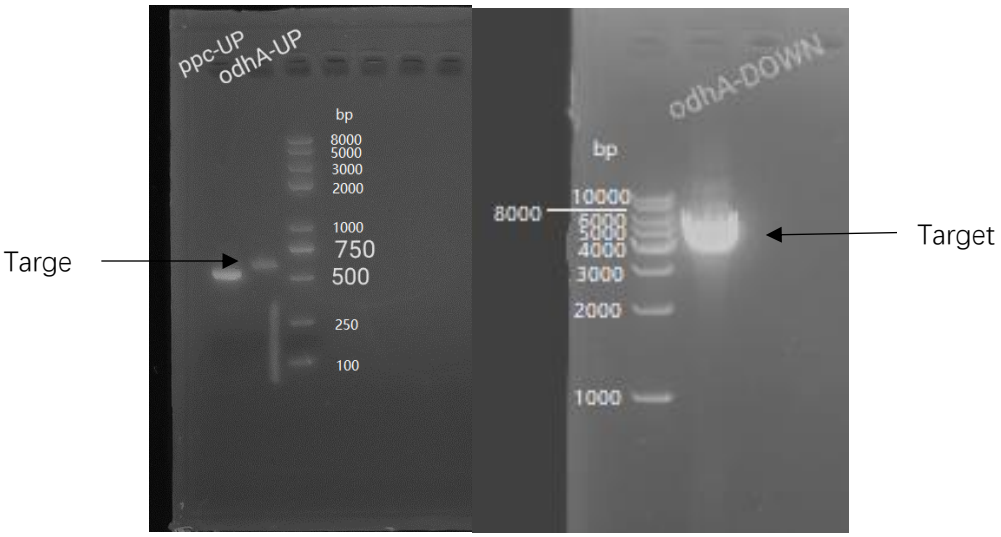
August 6th

Validation of PCR results for capB module product. Failed.

August 7th

1. Construction of recombinant plasmid pk18mobsacB-RBS-odhA

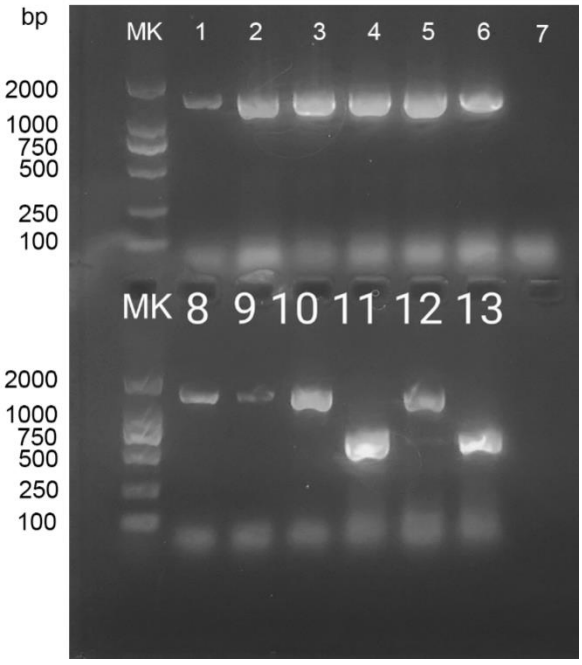
1.1. Amplification of odhA-UP & odhA-DOWN



1.2. Restriction digestion of pk18mobsacB

1.3. In-fusion cloning

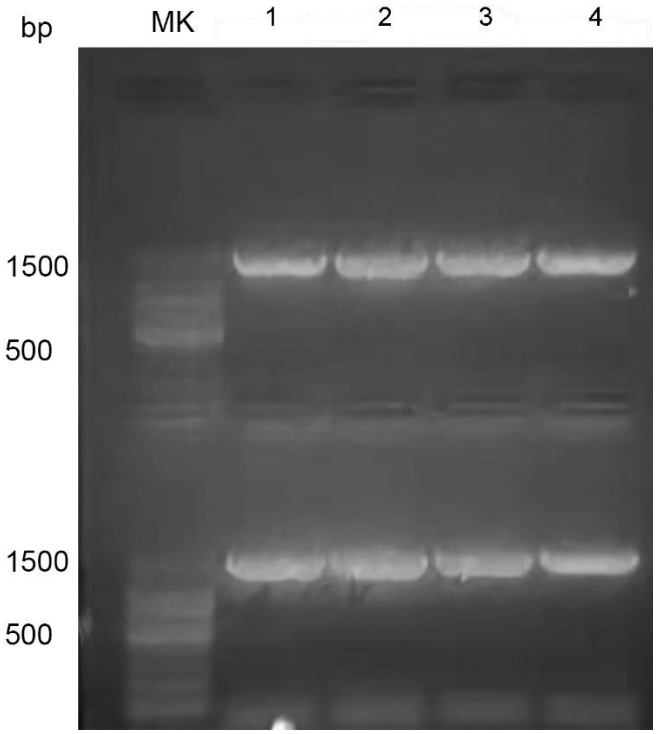
capB capC module build verification.

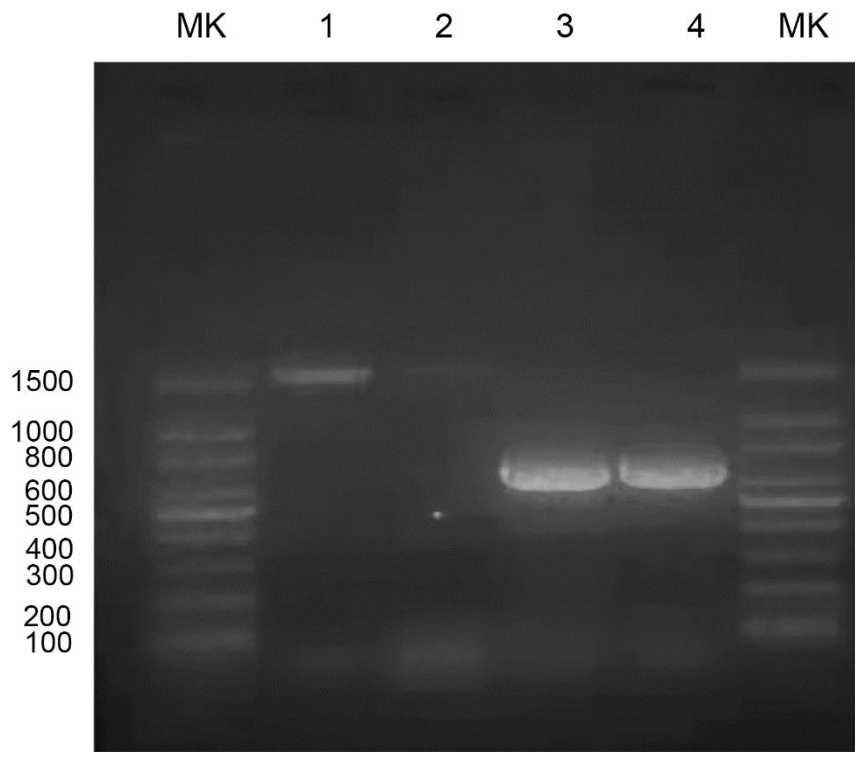


August 8th and 9th

PCR of ligated products was performed with the transformed bacteria of capA capB capC module.

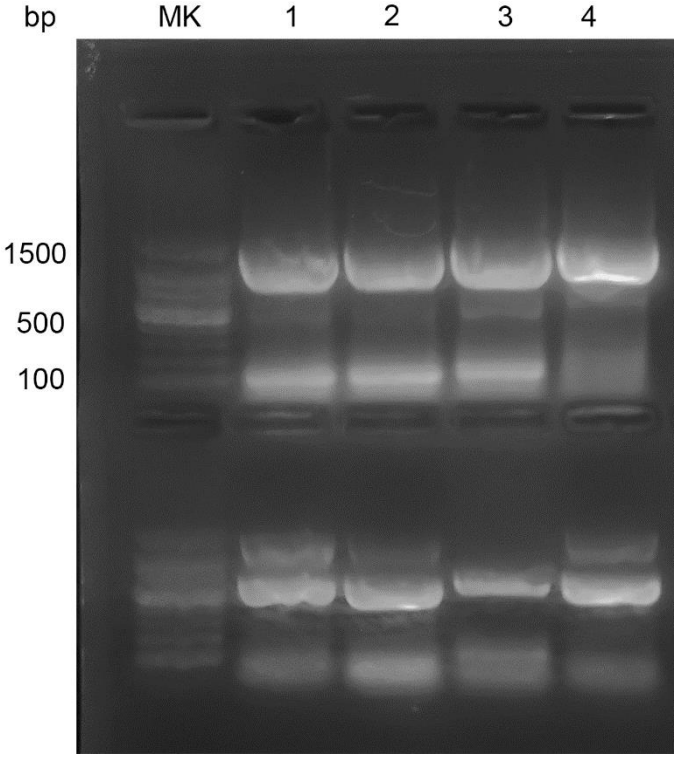
The capB capC PCR was repeated, except for the capC spurious bands, and the length was not correct, so the gum recovery was performed for 3. 25μL

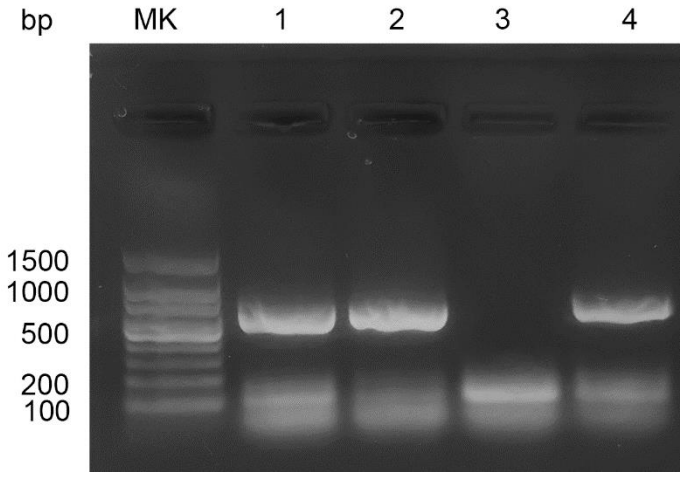




Gum recovery failed and string samples were taken.

The capB capC PCR was repeated, except for the capC spurious bands, and the length was not correct, so the gum recovery was performed for 3. 25μL



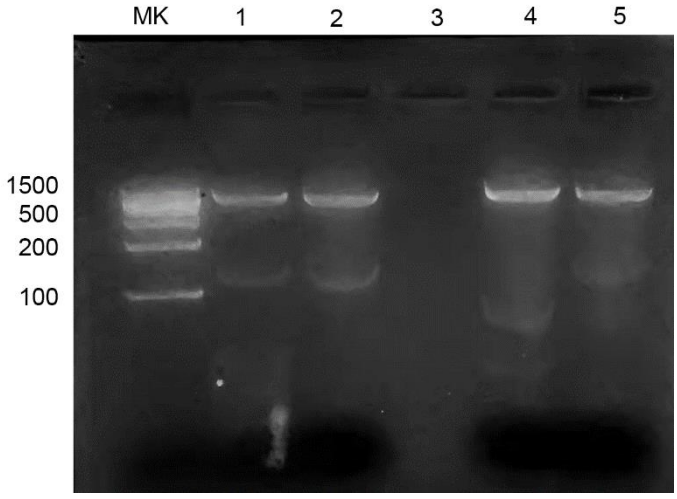


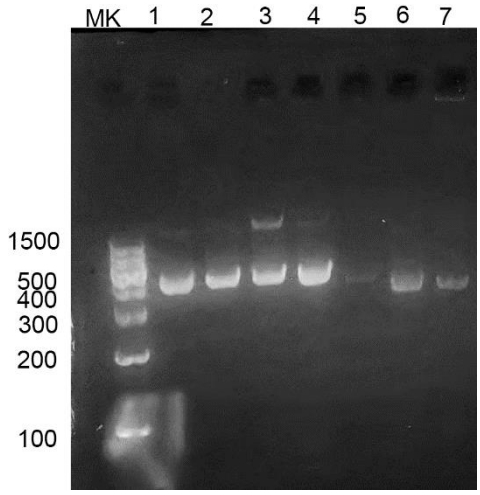
The subsequent PCRs all had a large number of spurious bands and the results were not very reliable.

1. Double enzyme tangent pXMJ19 (ECOR V, Xba I)
2. The plasmid pXMJ19-Patp2-GFP-rrnB was seamlessly cloned and transformed into E. coli DH5 α

August 10th

The transformed colonies were tested by PCR with enzymatic digestion assay.

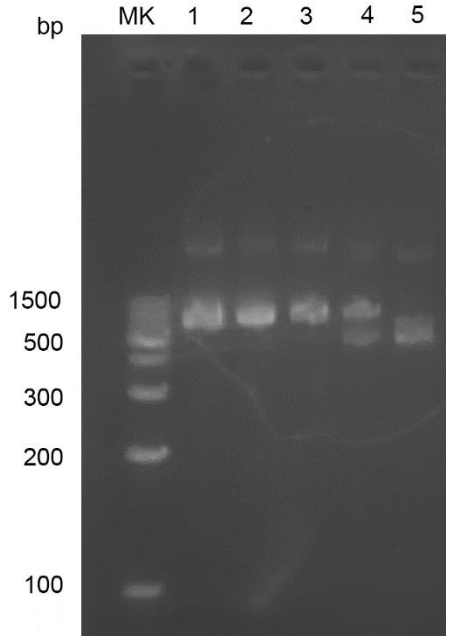




Transformation of DH5α

August 11th

capA capB capC seamless cloning plasmid extraction.

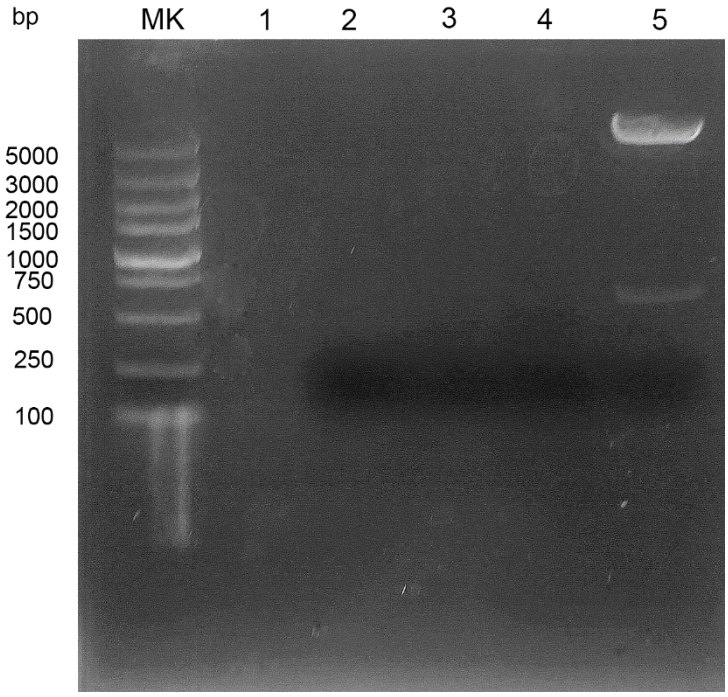


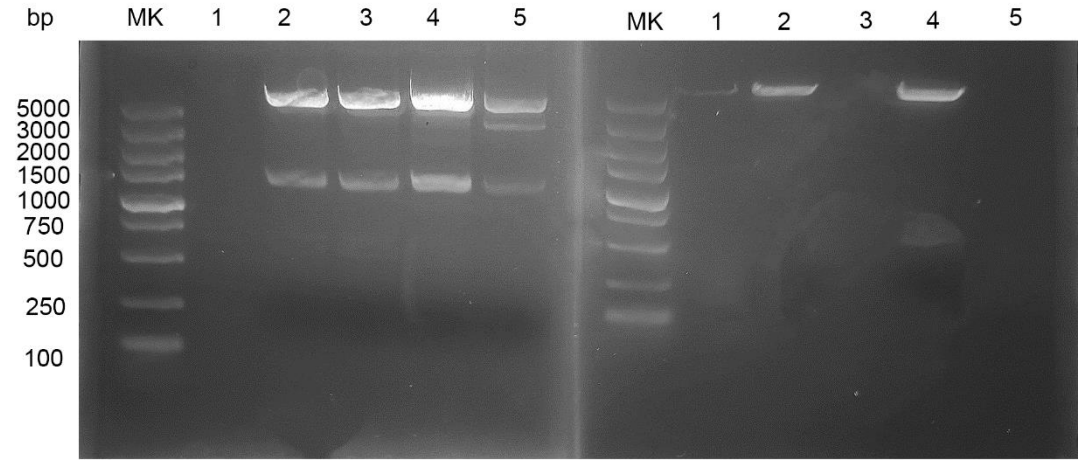
From left to right, lane 1 and 2 are module 1, lane 3 and 4 are module 2, and lane 5 is module 3.

Validation of enzyme digestion using EcoRI and HindIII
Bands are too faint and ready to be re-verified.

August 12th

Re-perform enzyme digestion verification.



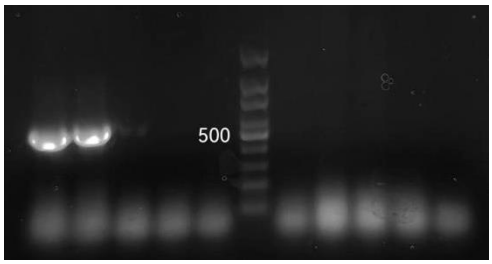


August 13th -20th

Plasmid pXMJ19-GFP was extracted, digested and sequenced.

Verification of recombinant plasmid

Colony PCR



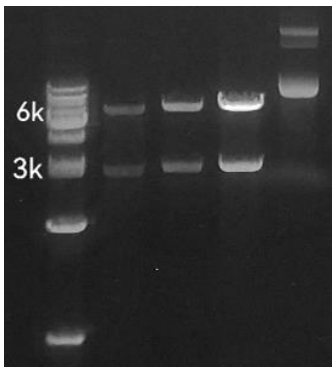
lane1:positive control;lane2-5:colony PCR;lane6:marker;lane7-10:colony PCR;lane11:negative control

Double digestion

The recombinant plasmid pk18mobsacB-RBS-ppc was digested by XbaI(NEB) and EcoRV(NEB).

rCutSmart buffer	2μl
Plasmid	5μl
XbaI	1μl
EcoRV-HF	1μl
ddH_2O	11μl

Incubate at 37℃ overnight.



lane1:marker;lane2-4:double digestion;lane5:pk18mobsacB-RBS-ppc

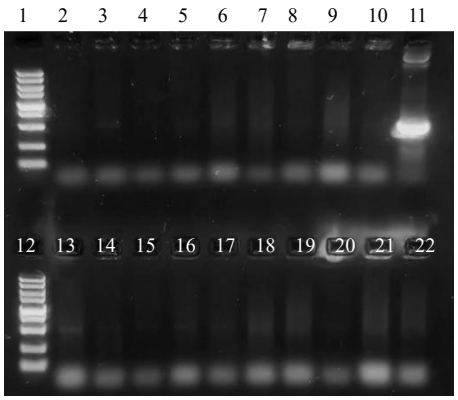
Sequencing

Preparation of *Corynebacterium glutamicum* ATCC 13032 competent cell

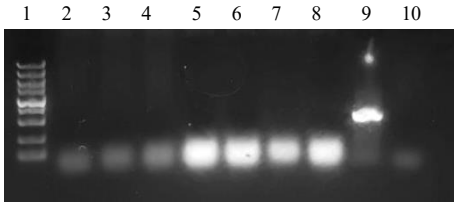
Transformation of *Corynebacterium glutamicum* ATCC 13032 by electroporation

Screening

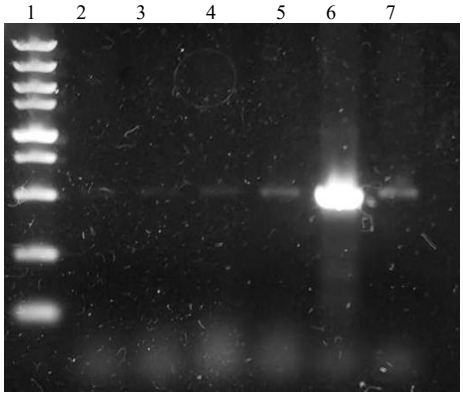
Colony PCR failure



lane1:Marker;lane2-10:colony PCR;lane11:positive control;lane12:Marker;lane13-21:colony PCR;lane22:negative control



lane1:Marker;lane2-8:colony PCR;lane9:positive control;lane10:negative control



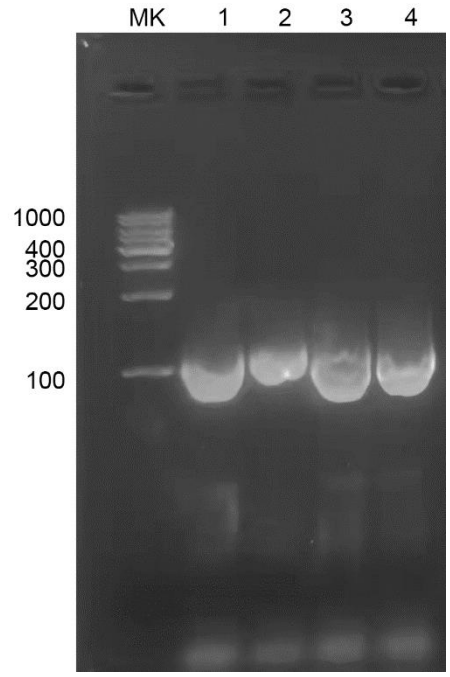
lane1:Marker;lane2-5:colony PCR;lane6:positive control;lane7:negative control

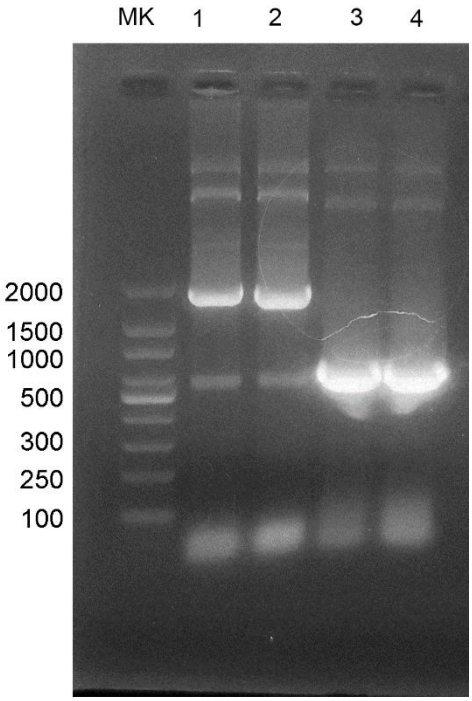
August 21st

Transformation of DH5α

August 23rd-25th

PCR amplification of capA capB capC ligated products, plus homology arms, followed by gel recovery

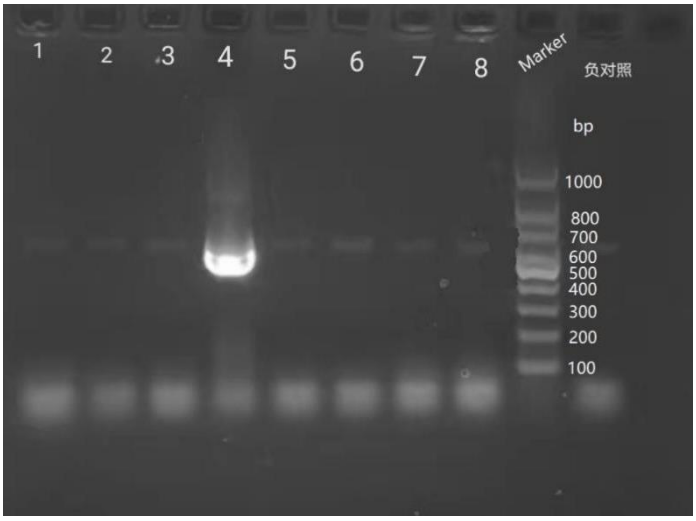




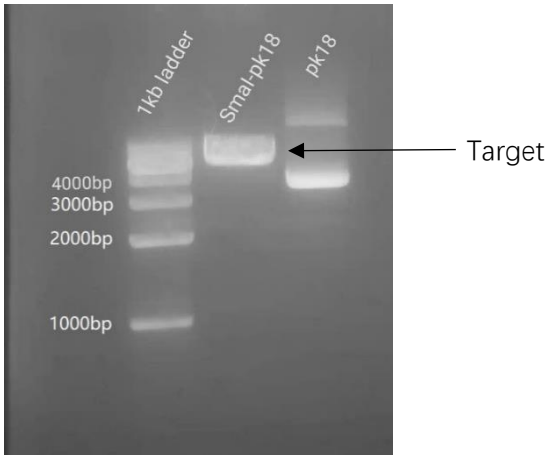
August 24th-28th

Verification of recombinant plasmid

Colony PCR



Double digestion



Sequencing

August 26th

Seamless cloning
Sequencing failed after transformation, it is empty pXMJ19

August 29th

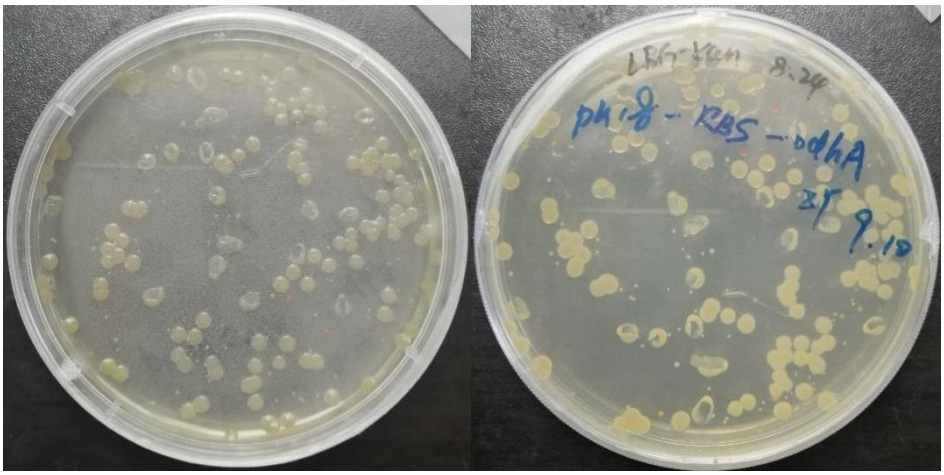
After communicating with BUCT team, considering the instability of seamless cloning, BUCT suggested us to use enzyme ligation method to ligate these three modules.
The capA capC module was added with the enzyme cut site using PCR.

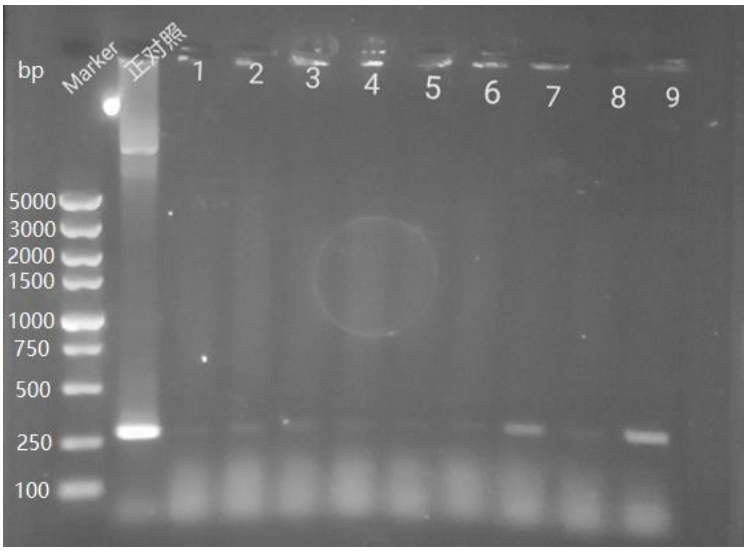
1. Plasmid pXMJ19-Patp2-GFP-rrnB was extracted, digested and sequenced.
2. Linearization pXMJ19 (Hind III)
3. The target gene ndoA was obtained by PCR
4. Seamless cloning and construction of plasmid pXMJ19 -ndoA

5. Plasmid pXMJ19 ndoa was extracted, digested and sequenced.

September 1st

Transformation of *Corynebacterium glutamicum* ATCC 13032





September 5th

1. Corynebacterium glutamicum atcc13032 was transformed by electrotransformation with plasmids pXMJ19-mazF and pXMJ19-ndoA.
2. The salt promoter Pgisb was obtained by PCR.
3. The plasmid pXMJ19-Pgisb2-GFP-rrnB was seamlessly cloned and transformed into E. coli DH5 α

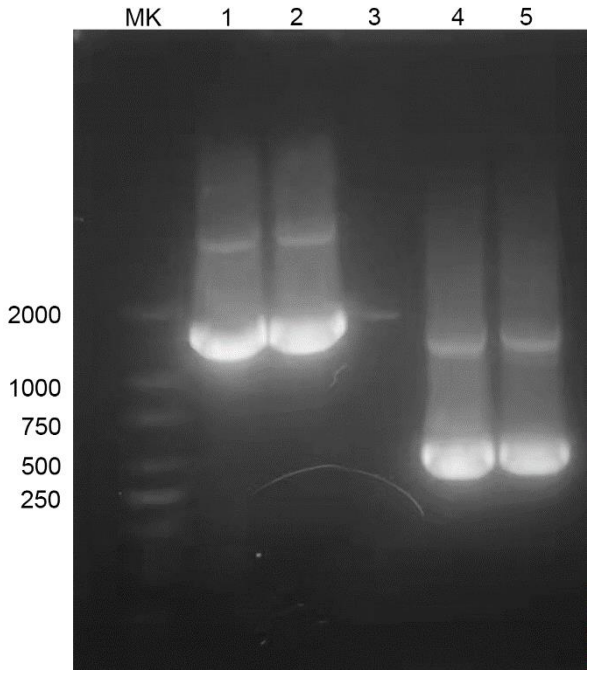
September 6th

1. Plasmid pXMJ19-Pgisb-GFP-rrnB was extracted, digested and sequenced.

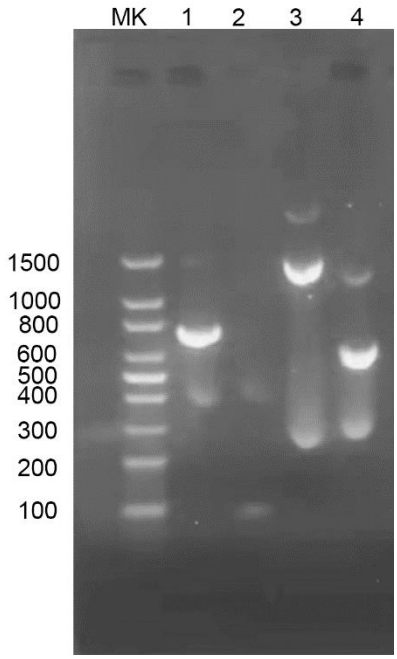
September 7th

capB enzyme cut site addition, using PCR approach.

The mCherry (K784041) gene was obtained from the plasmid with the addition of the homologous arm. p0864 plus the homologous arm linked to mCherry was used for PCR.



Gum recovery assay. The recovery solution was impure, resulting in abnormal behavior of the nucleic acid dye.

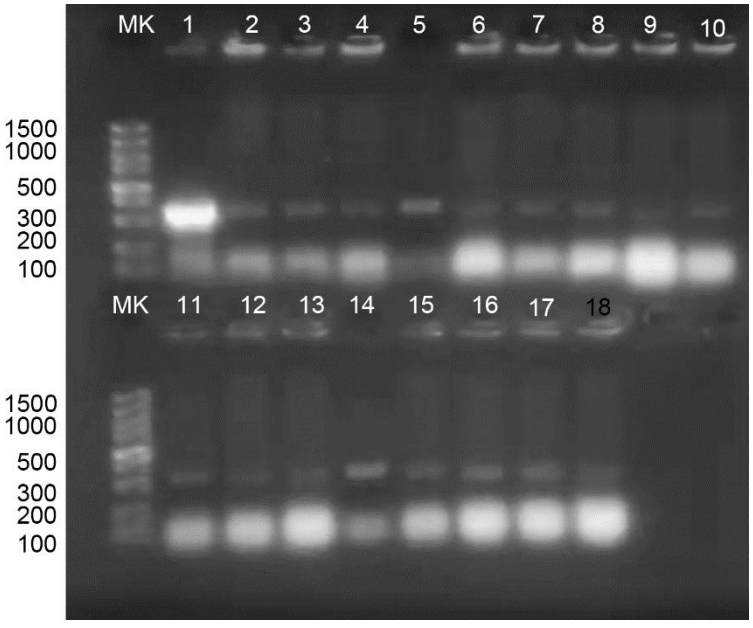


September 8th -14th

capA B C Enzyme ligation, transformation.

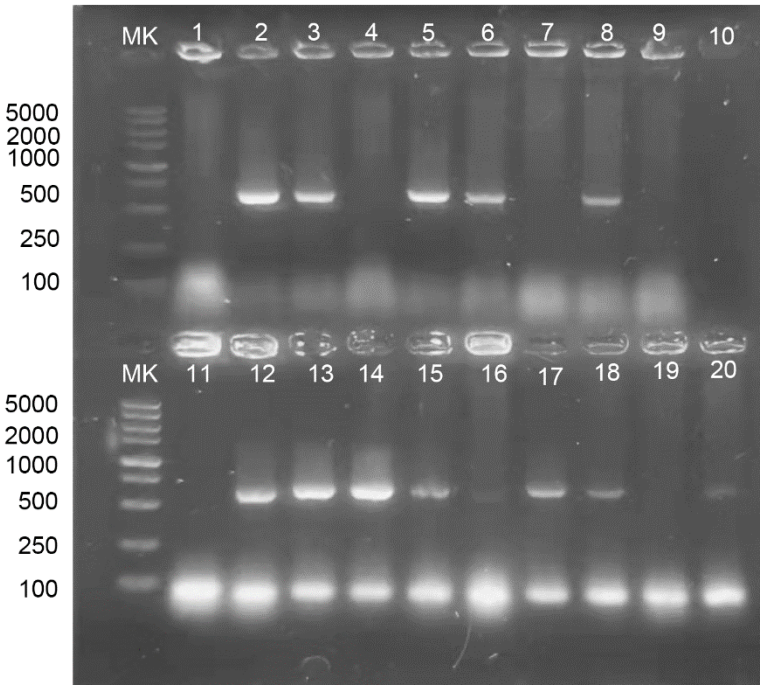
September 15th

The product colonies were identified by PCR. The control group also showed the target bands at the same time.



September 18th

The product colonies were continued for PCR identification. Everything that could lead to contamination was ruled out.

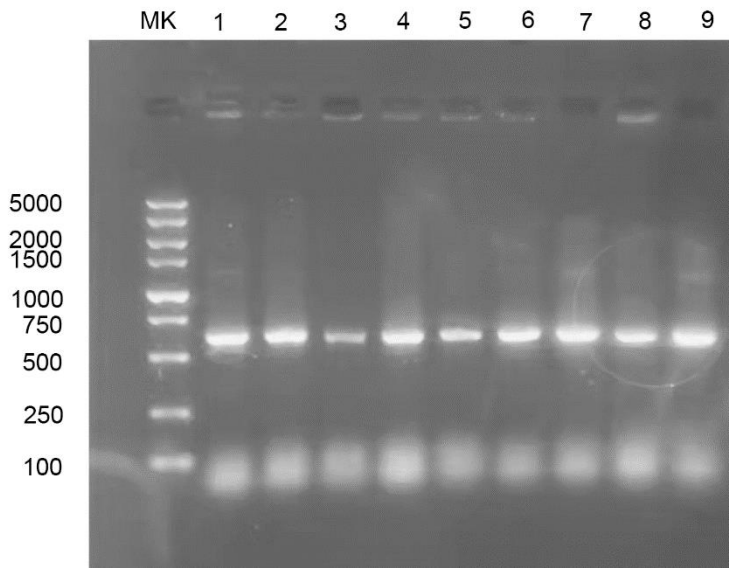


September 19th

1. Colony PCR validation of Corynebacterium glutamate transformants pxmj19 MazF and pxmj19 ndoa
2. Corynebacterium glutamicum ATCC13032 was transformed by electrotransformation using plasmids pXMJ19-Patp2-GFP-rrnB and pXMJ19-Pgisb-GFP-rrnB.
3. Lethal effect test of toxin gene ndoA

September 20th

P0864-mCHerry linkage product assay.



September 23rd

Product fluorescence detection.

September 24th

Identification of enzyme-linked products.

September 26th

PCR anti-odhA gene

There are two kinds of template sources: PCR from the extracted genome and colony PCR

The results of the experiment were as follows: during the preparation of agarose gel electrophoresis, the nucleic acid dye was forgotten to added so the band could not be observed after electrophoresis, and all samples were applied to the sample, so this experiment failed.

1. Lethal effect test of toxin gene ndoA
2. Colony PCR was used to verify the transformants pXMJ19-Pgisb-GFP-rrnB and pXMJ19-Patp2-GFP-rrnB of Corynebacterium glutamicum
3. Obtain the target gene lacIq by PCR

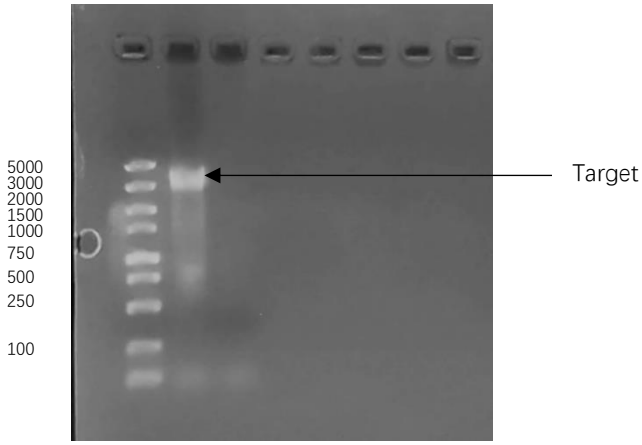
September 27th

PCR anti-odhA gene and verified by agarose gel electrophoresis

Results:

the PCR product samples are in lanes 2 and 3

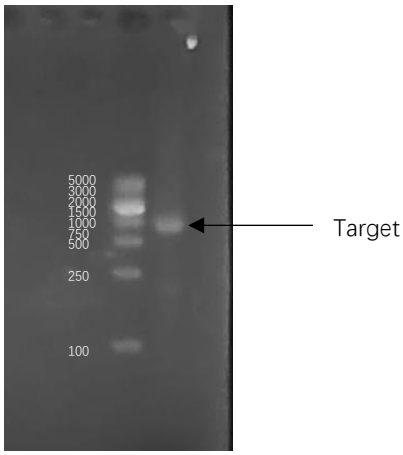
(bp) Marker/ sample



Gel Extraction

Results:

(bp) Marker/ sample



September 28th

The cleavage of plasmid and anti-odhA gene by restriction endonuclease（Pac I and Xbal）

Results：It is difficult to identify.

cleavage again

Results：Failed again.

October 2nd

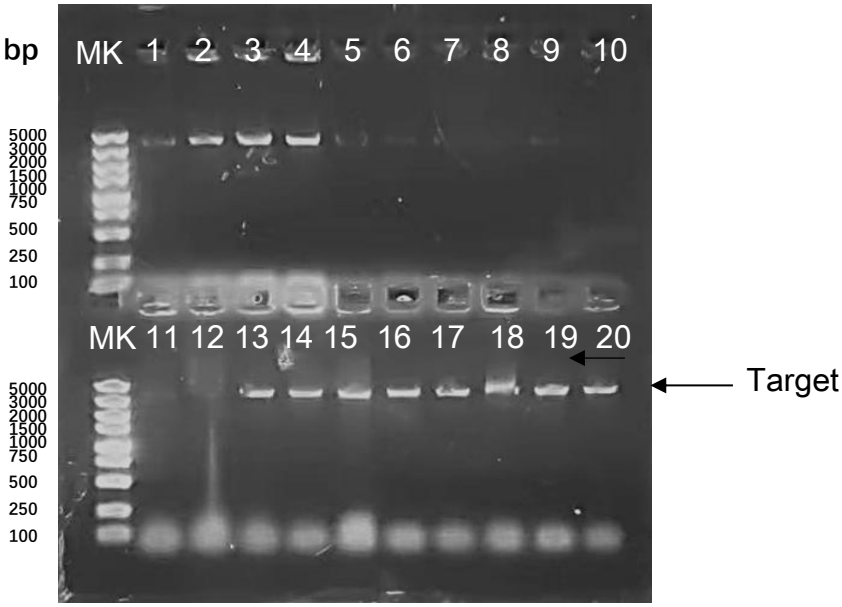
Transformed the linked product into E. coli and sreaded the plate

October 3rd

1. Lethal effect test of toxin gene ndoA
2. Effect test of saline alkali promoter Pgisb and Patp2
3. Construct plasmid pXMJ19-Pgisb-lacIq-rrnB by enzyme digestion

October 4th

Colony PCR（20 colonies）and verified by agarose gel electrophoresis



The stripe in lane 2,3,4,13,14,15,16,17,18,19,20 is correct and clear.

October 6th

The sequencing result showed that the some steps of the experiment we have done before were failed and we planned to transfer again.

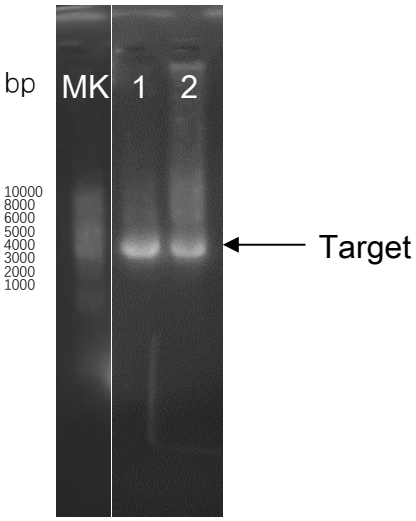
October 7th

Remove some of the colonies and put them into Test tubes containing LB(kana resistance) (AO14) and shake it overnight.

October 8th

Plasmid Extraction and then PCR anti-odhA gene

Results:



The results are shown in the lane 4 and 5.

Relaxation culture of Corynebacterium glutamicum and verification

October 10th

1. Construct plasmid pXMJ19-Patp2-lacIq-rrnB by enzyme digestion
2. Effect test of saline alkali promoter Pgisb and Patp2

October 10th-17th

1.Seamlessly clone to construct suicide track pXMJ19-Patp2-lacIq-rrnB-Pgisb-lacIq-rrnB-Ptac-lacIq-rrnB