5.31

Used PCR to verify the target sequences and synthetic primers. The PCR system is below.

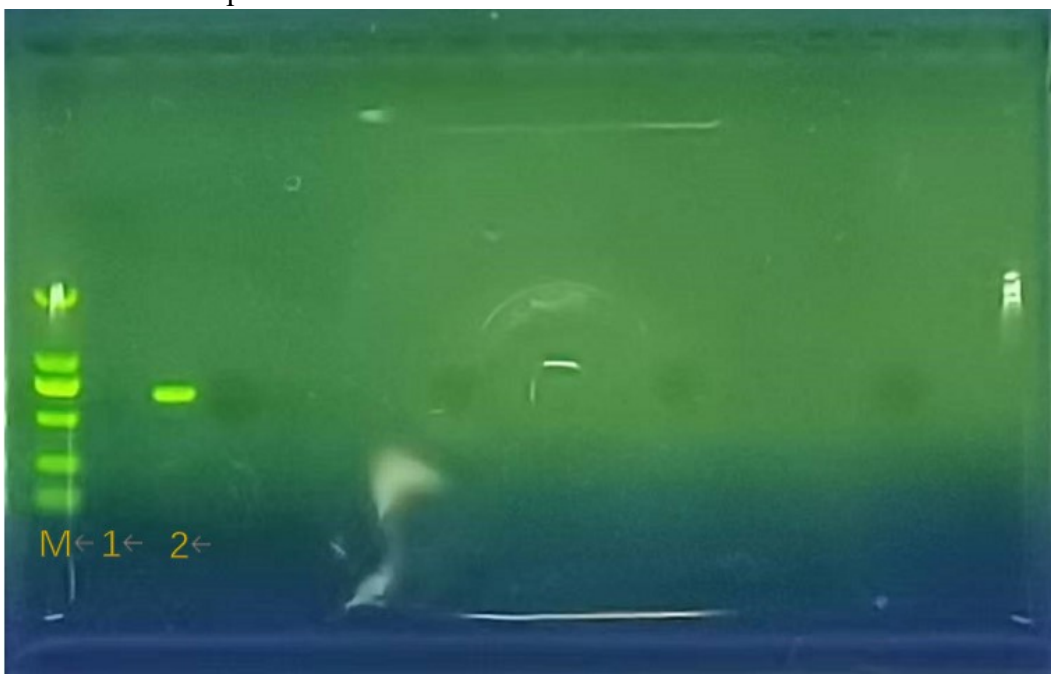
The reaction system of PCR

Composition of reaction system	Sample volume (μL)
Taq PCR MasterMix	25
Primer 1(anti-sense and sense primer)	1
Primer 2(anti-sense and sense primer)	1
ddH_2O	21
Genomic DNA	2
total	50

PCR reaction conditions:

1) Pre-denaturation at 95℃ for 5min;
2) Denaturation at 95℃ for 30s;
3) Annealing the primers at 54℃ for 30s;
4) The primer extended at 72℃ for 1 min;
5) Repeat step B-D 30 times;
6) Continue at 72℃ for 10min.

The gel diagram of target gene is as follows. No.1-2 refers to different products of PCR used different primers.



6.17

Recovery of BL12 strains. Activated the strains stored at 4℃ for preparation of competent cell. The strains were delineated on plates and incubated overnight at 37°C.

6.18
Prepared the materials for competent cell.

6.19
Preparation of competent cell.
1) Inoculate 50mL LB medium with 250μL of saturated overnight culture;
2) Shake at 37℃ until OD600=0.4 (usually 2-3 hours);
3) Place in an ice bath for 10mins;
4) Pre-cool solution, centrifuge, pipette tips, Eppendorf Tubes;
5) Transfer the culture into 1mL Eppendorf Tubes;
6) Centrifuge at 4000rpm for 5mins at 4℃;
7) Remove the medium, resuspend the cell pellet with 250μL 50mM $CaCl_2$ by swirling on ice gently;
8) Incubate on ice for 30 mins;
9) Repeat step 6;
10) Combine cells to one tube and add 40μL 18% glycerol;
11) Store in -80℃.

Urease
7.18
The genome of *Sporosarcina pasteurii* was extracted. And the required materials and experimental procedures are the same as July 8.
The final extracted genome was stored in -20℃refrigerator.
7.19
We determined the concentration of DNA extracted on 18 July.
The DNA was divided into three groups labeled as 2-1, 2-2 and 2-3 respectively, and its concentration was determined by OD value.

The OD values and concentrations of DNA

No.	A260/A280	A260/A230	Concentration(ng/ul)
2-1	2.123	1.601	154.25
2-2	2.171	1.837	158.8
2-3	2.16	1.653	151.95

The DNA of groups 2-1, 2-2 and 2-3 was amplified by long fragment PCR.
The required materials and experimental procedures are the same as July 9
7.21
Determination of urease activity as a function of time
Materials: urea solution 1.6M 175ml, bacterial solution 35ml
Urease activity was measured as a function of time.
Material: 1.6M 175ml urea solution , 35ml bacterial solution
We named blank LB medium with urea as blank, and the medium inoculated with bacteria and urea placed in the incubator as No.1, and the medium placed in the refrigerator with urea as No.2.

No.	Time interval(min)	Electrical conductivity
blank	0	3.05

	25	3.03
	10	3.03
	32	3.02
	28	3.01
	27	3.01
	15	3.01
	21	3.01
	0	7.31
	10	8.03
	10	8.32
	10	8.70
	20	9.41
	20	10.18
1	20	10.79
	20	11.44
	20	12.11
	20	12.75
	20	13.37
	20	13.99
	0	9.03
	10	9.71
2	10	10.48
	10	11.17

No. 2 test exceeded the instrument range after more than 30 minutes.

CA

7.26

(1) E. coli with recombinant plasmid of mASCA fragment and empty plasmid of petDuet-1 were resuscitated.

(2) Preparation of Amp+ liquid and solid LB medium.

(3)Amp+ solid LB medium was prepared as 200ml culture medium and 200ml Amp. Amp+ liquid LB medium was prepared as 200ml culture medium and 200ml Amp, of which 10ml was placed in the test tube and the rest was placed in the conical flask

(4) After sterilizing the culture medium, the resuscitated bacteria were inoculated and continued to be cultured

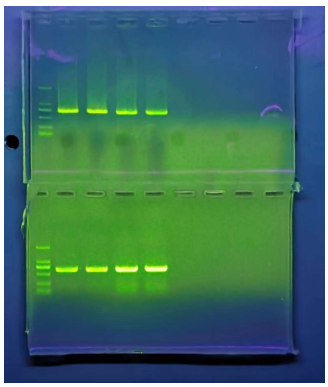
7.27

(1) The bacteria containing mASCA fragment were amplified by PCR.

The reaction system of PCR

Composition of reaction system	sample volume(μl)
2×Taq PCR MasterMix	25
sense primer	1
anti-sense primer	1
ddH2O	1.5
Template	21.5

We didn't get the result we expected. We reinoculated a tube of bacteria containing the original mASCA fragment and continued the culture. Then, the bacteria containing mASCA fragment and petDuet-1 plasmid were amplified by PCR, and the amplified products were detected by agarose gel electrophoresis.
The result is shown in figure:



The upper part is stained with GVII(500μ6×loading buffer+500μddw+3μGVII dye stock solution) during electrophoresis, and the lower part is stained with red fluorescent dye. The leftmost band is maker, and the bands from left to right are named as No.1, No.2, No.3, and No.4. And No.1 and No.2 are the bands measured by the bacterial solution, No.3 and No.4 are the bands measured by the bacterial colony.
(2)Glue cutting and recycling

No.	Concentration(ng/ul)
1	0.13
2	0.10
3	0.11
4	0.13

390μl TE was added to No.1 and No.4, and 300μl TE was added to No.2 and No.3.
(3) According to the instructions of epPCR, the No.2 tube was diluted 10 times to obtain 1ng/ μL, which was used as epPCR template together with the stock solution.

7.29
Error-prone PCR of the mASCA gene fragment.
Extraction of pETDuet-1 for genes obtained by error-prone PCR.
Plasmid Miniprep Kit

Expected number of mutations：6。

System: 30μL

Concentration of template	1ng/μL	10ng/μL
Mix	3	3
dNTP	3	3
MnCl2	4	4
dGTP	2	2

template	1	1
Primer	1×2	1×2
Taq	0.5	0.5
ddwater	14.5	14.5

The cyclic settings for PCR：（×45）

94℃ 3min
94℃ 1min
45℃ 1min （×45）

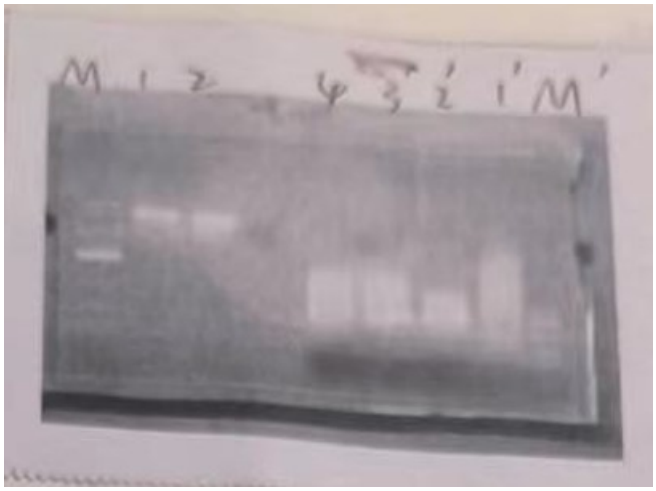
72℃ 1min

plasmid extraction：Use Plasmid Miniprep Kit to extract pETDuet-1.

Then we used Gel Extraction Kit to extract gel and the following weight.

	Number	The weight of gel(g)
Plasmid	1	0.10
Plasmid	2	0.09
mASCA(10ng/μL)	3	0.09
mASCA(10ng/μL)	4	0.04

Those can be used in transformation.



M: DL10000
M’: DL2000
1,2: pET Duet-1
1’,2’:epPCR result (1ng/μL of template)
3,4: epPCR result (10ng/μL of template)

7.30
mASCA was double enzyme digested and connected and transformed.

Double enzyme digestion liquid ratio：

	PCR product(μL)	plasmid(μL)
DNA	20	25

Buffer	5	5
HindIII	1	1
EcoRI	1	1
ddwater	23	18

80℃ 20min after acting 37℃ 1h.

8.2

mASCA Gel Extraction failed.

We succeeded in mASCA PCR, the concentration of PCR product was 26.33ng/μL. Then we diluted it as the template of following epPCR.

We failed in mASCA kit miniprep and epPCR with obscure strips in electrophoresis. The following is the result of electrophoresis.

The concentration of gel extraction:

	1(ng/μL)	2(ng/μL)
A	27	16.3
B	25.7	16.3
C	26.3	16.6
Average	26.3	16.4

And we failed in kit miniprep without reliable results.

8.3

Using the plasmid extracted by kit before to start mASCA epPCR.

We succeeded in plasmid miniprep of mASCA epPCR.

We adjusted epPCR cycling numbers from 45 to 35.

And we finally had good results and found it was suitable for shaking flasks lasting for 24-36h.

8.4

We started double enzyme digestion, gel extraction, connection and cells transformation.

And we stayed half of connection plasmid overnight and the other half connecting 10min.

The following is products of enzyme digestion:

M: 2000 marker
1: ep PCR mASCA
2: after enzyme digestion
3: after enzyme digestion
4: pETDuet-1
M': 10000
The connection system(20μL)

	μL
Buffer	2
Vector	8
Insert	6
ddwater	3
T4 ligase	1

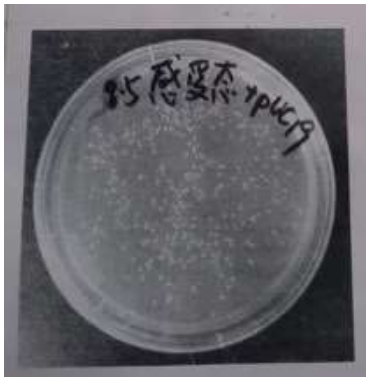
20min at room temperature and then react at 65℃ staying 10min.
Then we used 5μL competent cells to connect but failed with no bacterial colony.

8.3
pcr products were digested by plasmid, recovered by gel cutting (not photographed), and ligated overnight at 16 ° C

8.4
Conversion of ep masca
Connect an enzyme at room temperature for 20min, and convert it with overnight

8.5
Preparation of competent cells, ligature transformation (not grown)
The competent cells were remanufactured and the gel recovered products were ligated again.

8.6
Convert defeat
But competent cells with puc19 grew



8.8
1.Msaca ep PCR, power failure occurred during the experiment
2. Plasmid extraction (not extracted)
3.ep PCR glue cutting recovery
4. EpPCR, original PETduet-1 digestion and ligation transformation

8.9
1. No plasmid was transferred on August 8
2. 8.8 Recovery of the remaining enzyme digestion product glue
3.Using the newly arrived t4 ligase, the transformation was done with the new enzyme

8.10
1. Failed to convert on August 9
Plasmids were extracted, plasmids +8.8ep mASCA were digested, plasmids were run to check whether there was plasmids (there were no plasmids), and ep PCR products were digested again
3. Transform today
(1) Self-ligating after p ET Duet digestion
(2) epmASCA +pETDuet-1 connection
(3) P etduet-1
(4) pEt Duet-1 was digested and self-ligated

8.11
Yesterday the result
p ET Duet-1 Imported successfully
p ETDuet-1 epm ASCA import failed
p ET Duet-1 Imported successfully
p ET Duet-1 Failed to import epmASCA
Description:
(1) The plasmid was no problem
(2) No problem with at least one endonuclease
(3) No problem with T4 ligase
(4) The receptive state is fine

8.12

The ep PCR was redone

Plan: Hind III, EcoRI each enzyme digestion plasmid transformation, to verify whether the enzyme is active

8.13 Carbonic Anhydrase

Enzymatic digestion of the pETDuet-1 plasmid:

	①	②
vector	25	25
buffer	5	5
Hind III	1	1
ddH_2O	18	18

8.18 Carbonic Anhydrase

epPCR was performed on plasmid pETDuet-1:

Attempt to reduce the expected number of mutations given previous conversion failures.

Expected number of mutations：5

System：30μL

Concentration	1ng/μL	10ng/μL
Mix 10×	3	3
dNTP	3	3
$MnCl_2$	4	4
dG	1	1
Template	1	1
pF	1	1
pR	1	1
Taq	0.5	0.5
ddH_2O	15.5	15.5

8.18 Urease

Extraction of the bacteriophage genome.

Elution was carried out using pure water and CE Buffer respectively：

Pure Water	CE Buffer
2-1：47.650 ng/μL	1-1：30.900ng/μL

2-2：54.850 ng/μL	1-2：32.150ng/μL

Electrophoresis results: no bands
Nucleic acid dye sensitivity 50ng, 90-150ng added to sample

Amplification of bacterial broth with solid media:
Flat coating: 2
Planar Scribing: 2

8.19 Urease
Extraction of the bacteriophage genome.
Elution was carried out using CE Buffer.
Extend the 37°C water bath to 1 hour after the addition of lysozyme.

Nucleic acid concentration: ①119.70 ng/μL ② 71.450 ng/μL

Electrophoresis results:
1. After electrophoresis, three lanes with bands (including 10,000 marker) are clearly visible under UV light, and the trailing tail is almost invisible.
2. The electrophoresis time is too long, no effective result.

8.20 Urease
PCR of the urease E and F subunit genes.
(1)Second primer
System:

	①	②
pF	2.5	2.5
pR	2.5	2.5
Template DNA	8	12
Q5 High-Fidelity 2× Master Mix	25	25
Nuclear-Free Water	12	8

Thermocycling Conditions For PCR:

STEP	TEMP	TIME
Initial Denaturation	98℃	30s
25-35 Cycles	98℃	10s
	72℃	5s
	72℃	10s

Final Extension	72℃	1min
Hold	4℃	∞

Electrophoresis results: Light bright bands of about 500bp in both lanes.

(2) Third primer

Conditions as above

Electrophoresis results: ①The template PCR product has a light bright band of about 750-1000 bp in the lane.

②No band in the lane where the template PCR product is located.

8.21 Urease

PCR using Phanta Mix to amplify a 1700bp fragment

System:

Master Mix	25μL
pF(10mM)	2μL
pR(10mM)	1μL
Template	10-500ng(1-2μL)
ddH_2O	Up to 50μL

Thermocycling Conditions For PCR:

Predenaturation	98℃	30s	
Denaturation	98℃	10s	35cycle
Annealing	T_m[a]	5s	
Extension	72℃	10s	
Final Extension	72℃	1min	
Hold	4℃	∞	

T_m[a]: 56.6℃, 58.6℃, 60.0℃, 63.1℃, 65.2℃

8.21 Urease
Extraction of the bacteriophage genome.
1. PCR with third primer
System as 8.20
Result: A bright band slightly over 2000bp, suspected to be the target product.

2. Extraction of the bacteriophage genome with methods as 8.19.
(1) Morning:

①34.250 ng/μL

②13.250 ng/μL

(2) Afternoon:

①31.700 ng/μL

②16.600 ng/μL

③22.100 ng/μL

④After centrifugation, large particles of material were found to be clogged, suspected to be incomplete disintegration of the organism, and were discarded.

8.27 Urease
Extraction of the bacteriophage genome.
Elution was carried out using CE Buffer(Lysozyme treatment 1h).
Nucleic acid concentration:

1: 130.10 ng/μL

2: 123.10 ng/μL

3: 66.250 ng/μL

4: 220.75 ng/μL

Result: Heavy dragging.

8.28
Whole genome amplification.

Using annealing temperature gradients of PCR: 57.5℃, 58.6℃, 60.0℃, 61.7℃,

63.1℃

to finding the optimum annealing temperature.

Result: Amplification error

Passage culture of Bacillus subtilis in solid medium at 30°C.

9.23
Colony PCR on colonies from EP1-EP7.

System: 20μL

Mix(2×)	10μL
Template	1μL
DNA Pol	0.5μL
pR	1μL
pF	1μL
ddH_2O	16.5μL

Thermocycling Conditions For PCR:

Predenaturation	98℃	30s	

Denaturation	98℃	5s	35cycle
Annealing	62	5s	
Extension	72℃	20s	
Final Extension	72℃	1min	
Hold	4℃	∞	