Notebook September

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Date 8.29

Plasmid Extraction of mtr-part

Recorder: Liwen Zhang

1.Centifuge 1.5 mL bacterium solution at 11000 rpm for 2 minutes. Remove the supernatant. Repeat twice.

2.Add 250 μL Buffer P1, resuspend cells.

3.Add 250 μL Buffer P2, mix well, 3 min's standing.

4.Add 350 μL Buffer P3, mix well.

5.13400 rpm centifuge 10 min, move all supernatant to adsorption column, 11000 rpm centifuge 60 s, discard filtrate.

6.Add 500 μL Buffer DW1, 12000 rpm centifuge 60 s, discard filtrate.

7.Add 500 μL Wash Solution, 12000 rpm centifuge 60 s, discard filtrate. Repeat once.

8.12000 rpm centifuge 1 min.

9.Lying for 10 min.

10.Put the adsorption column in a new EP tube. Add 50 μL 50°C ddH2O, 10 min's standing, 12000 rpm centifuge 1 min. Preserve in the EP tube with ddH2O at 4 degree Celsius.

After extraction, we measured the OD A260/A280, OD A260/A230 and the density of plasmids. The results are shown in the chart below.

sample	**mtr-part 4**	**mtr-part 6**	**mtr-part 7**
Concentration(ng/μL)	423.8	328.3	405.9
260/280	1.83	1.85	1.85
260/230	1.84	2.17	2.12

Date 9.9

Bac PCR of Mtr and Ccm Recorder: Wenfei Yu

Experimental materials

1. Template: Mtr+Ccm in E.coli;
2. Primer: T7,T7-TER,VF2,VR. Designed by ourselves, synthesized by Sangon Biotech;
3. Sterilized ddH2O, Primerstar.

Procedure:

1.Prepare 8 PCR tubes and sequentially add：

sample	**1**	**2**	**3**	**4**	
Sterilized ddH2O	7 μL	7 μL	7 μL	7 μL	
2×Taq	10 μL	10 μL	10 μL	10 μL	10 μL
template	1 μL	1 μL	1 μL	1 μL	
VF2	1 μL	1 μL	1 μL	1 μL	
VR	1 μL	1 μL	1 μL	1 μL	

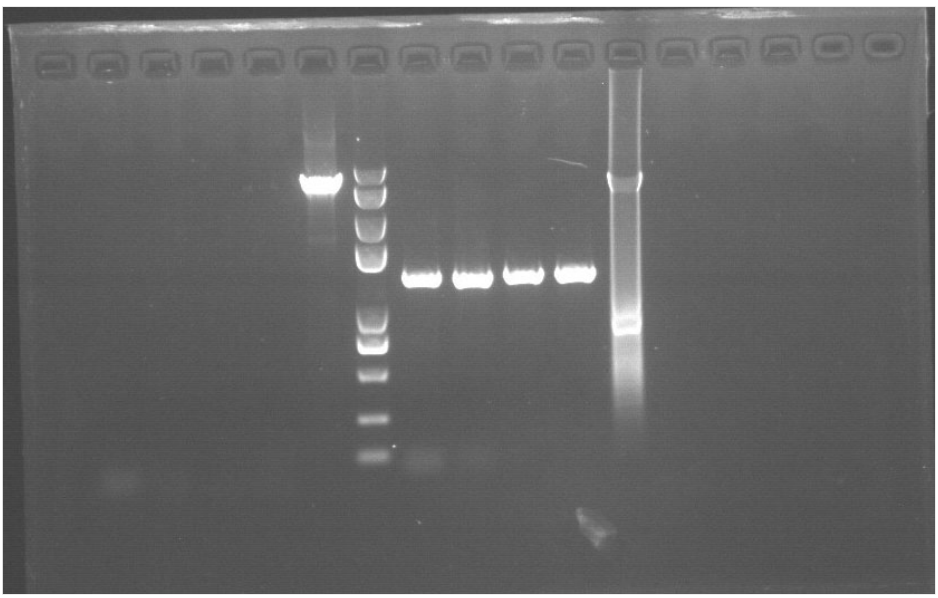
sample	1	2	3	4
total	20 μL	20 μL	20 μL	20 μL

sample	5	6	7	8	
Sterilized ddH2O	7 μL	7 μL	7 μL	7 μL	
Primerstar	10 μL	10 μL	10 μL	10 μL	10 μL
template	1 μL	1 μL	1 μL	1 μL	
T7	1 μL	1 μL	1 μL	1 μL	
T7-TER	1 μL	1 μL	1 μL		
total	20 μL	20 μL	20 μL	20 μL	

2.PCR reaction 1~4 |stage|temperature|time| |-| |step 1|98|10 min| |step 2|98|10 s| |step 3|47|30 s| |step 4|72|5 min 30 s| |step 5|72|10 min| |step 6|4|-- 30 cycles(step 2 ~ step 4)

5~8 |stage|temperature|time| |-| |step 1|95|10 min| |step 2|98|5 s| |step 3|56|15 s| |step 4|72|7 min| |step 5|72|10 min| |step 6|4|-- 30 cycles(step 2 ~ step 4)

3.Agarose gel electrophoresis Result:



(From left to right: 5~8;pcr of Ccm;Marker Q;1~4 ;pcr of Mtr) **Date 9.24**

Confirmation of Ccm A-H's function

Recorder: Shihan Zhu & Chenyang Li & Yonghao Liang

1. Template: Strain BL21 contains both Mtr gene and Ccm A-H gene;
2. Primer: T7, T7-ter, VF2, VR, Mtr-f, Mtr-r. Designed by ourselves, synthesized by General Biosystems;
3. Sterilized ddH2O, 2×PrimeStar(Premix), bought from Takara.

Bac PCR Procedure: 1.Prepare 6 PCR tubes and sequentially add：

sample	1	2	3	4	5	6
Sterilized ddH2O	7 μL	7 μL	7 μL	7 μL	7 μL	7 μL
2×Prime Star(premix)	10 μL	10 μL	10 μL	10 μL	10 μL	10 μL
template	1 μL	1 μL	1 μL	1 μL	1 μL	1 μL
primer 1	1 μL	1 μL	1 μL	1 μL	1 μL	1 μL
primer 2	1 μL	1 μL	1 μL	1 μL	1 μL	1 μL
total	20 μL	20 μL	20 μL	20 μL	20 μL	20 μL

2.PCR reaction 1,2,3,4 Parameters setting：(for PCR of Mtr)

stage	temperature	time
step 1	95	10 min
step 2	98	5 s
step 3	56	5 s
step 4	72	5 min 30 s
step 5	72	10 min
step 6	4	--

30 cycles(step 2 ~ step 4)

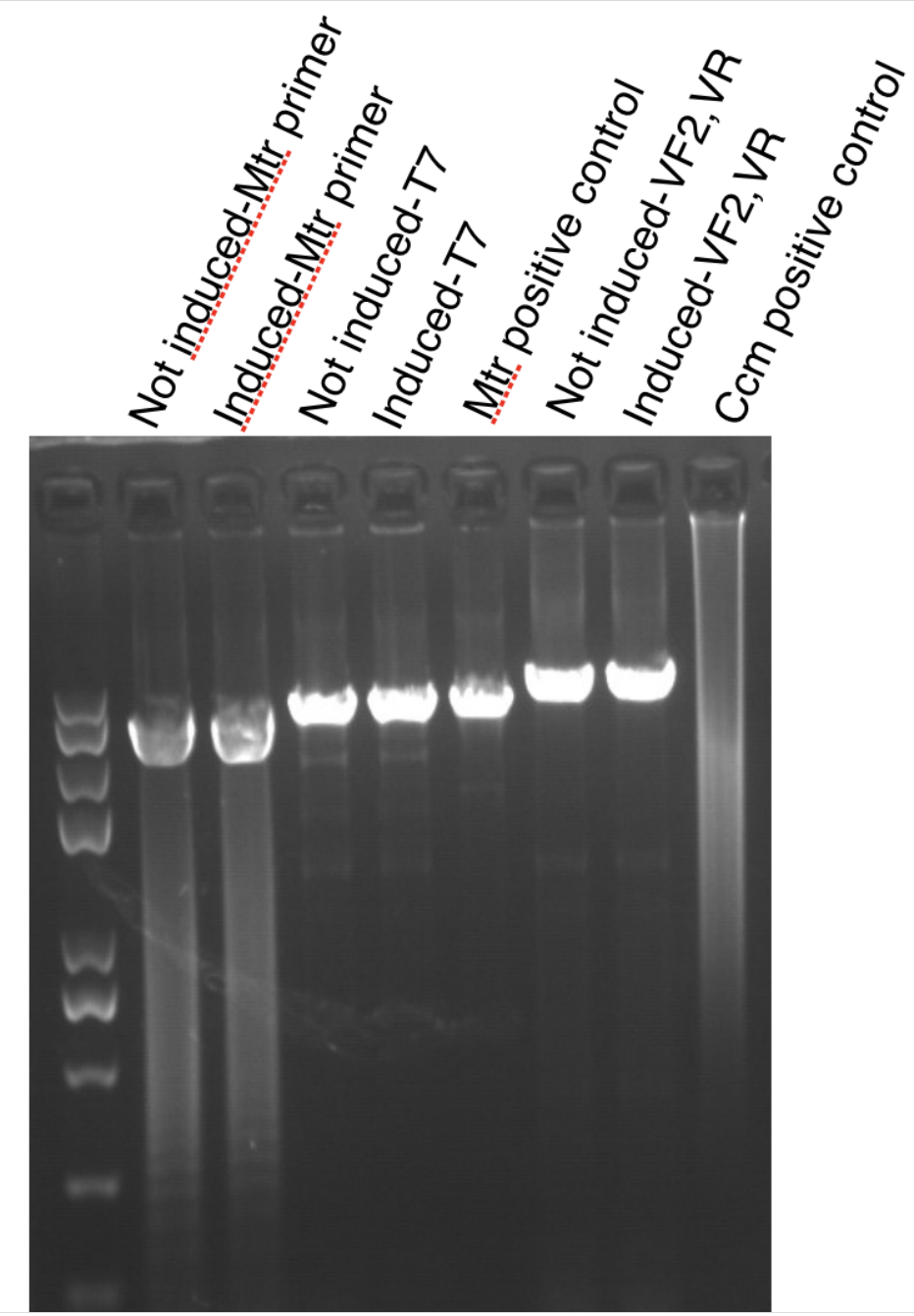
5,6 Parameters setting：(for PCR of Ccm A-H)

stage	temperature	time
step 1	95	10 min
step 2	98	5 s
step 3	56	5 s
step 4	72	7 min
step 5	72	10 min
step 6	4	--

30 cycles(step 2 ~ step 4)

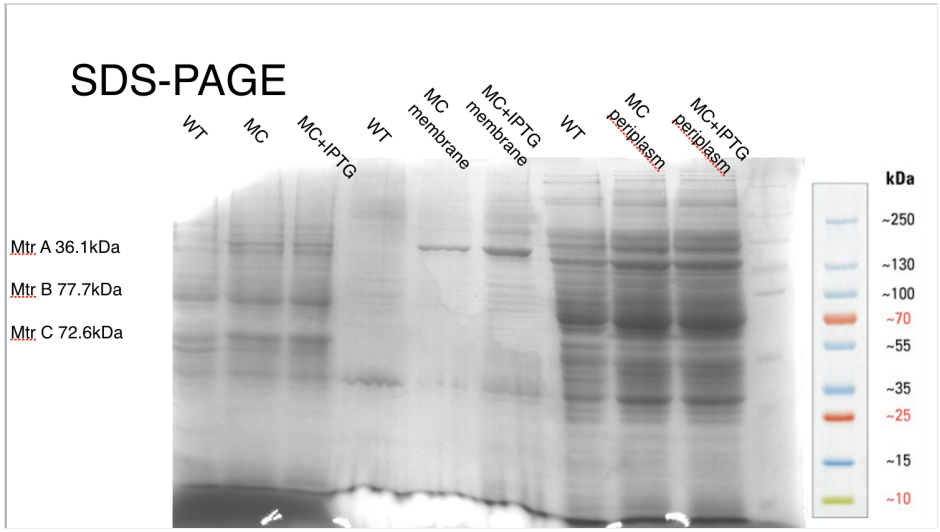
帮助

Result:



SDS-PAGE of Mtr Procedure: 1.Inoculate the bacteria from the glycerinum stock and cultivate it overnight, 37℃, 250 rpm; 2.Delute the bacteria into a 5 mL medium and cultivate for 6 hours, 37℃, 250 rpm; 3.Dulute 2 mL of the bacteria above into a 200 mL medium and cultivate it till its OD reach 0.4-0.6; 4.Add appropriate concentration of inducer into the system and induce the expression for 1-3 hours; 5.Take certain volume of bacteria out of the medium, mix with appropriate volume of protein loading buffe, and heat it at 100 ℃ for 20 mins; 6.Use the heated sample for SDS-PAGE.

Result:

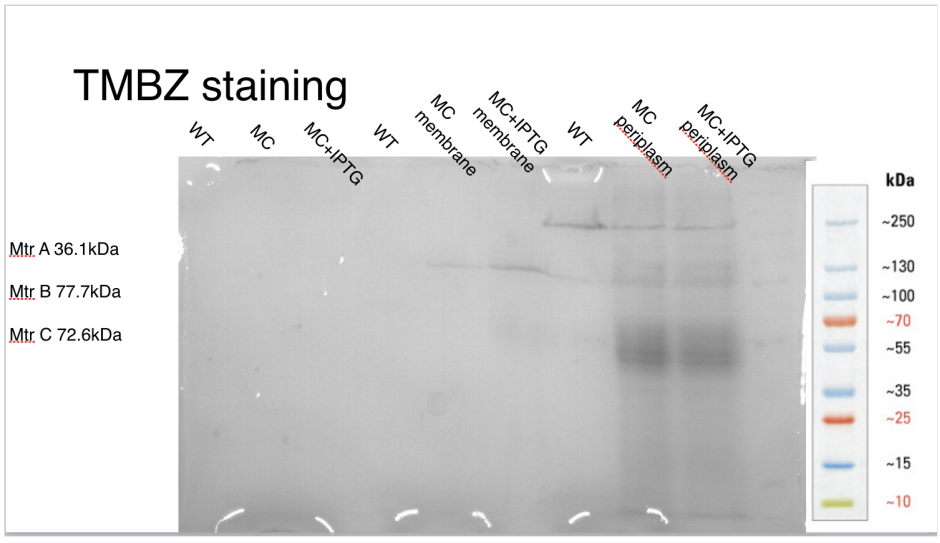


Heme Staining

Procedure:

1. Solutions you need for the stain:
   - 6.3mM TMBZ in methanol: 60 mg TMBZ in 30mL methanol.
   - 0.25M sodium acetate, pH 5.0
   - 30% hydrogen peroxide (9.79M, in the fridge)
   - Isopropanol
2. Make the TMBZ solution while the gel is running. This takes time to solubilize. Co
3. Rinse the gel with water for 5 minutes
4. Immediately before use, mix 30mL TMBZ solution with 70mL sodium acetate solution (
5. Immerse the gel in the solution above. put gel in the dark. (ie a drawer)
6. Mix occasionally (every 15 minutes) for 1-2 hours
7. Add 495 µL 30% hydrogen peroxide (final concentration 30 mM in 100 mL). Mix well.
8. Staining should be visible within 3 minutes and increases in intensity over the ne
9. Background of the gel may be removed by destaining with 3:7 isopropanol:0.25M sodi

Result:



Western Blot of Mtr

Procedure:

1. Block the membrane for 1 h at room temperature or overnight at 4°C using blocking buffer.
2. Incubate the membrane with appropriate dilutions of primary antibody in blocking buffer. We recommend overnight incubation at 4°C; other conditions can be optimized.
3. Wash the membrane in three washes of TBST, 5 min each.
4. Incubate the membrane with the recommended dilution of conjugated secondary antibody in blocking buffer at room temperature for 1 h.
5. Wash the membrane in three washes of TBST, 5 min each.

6. For signal development, follow the kit manufacturer's recommendations. Remove excess reagent and cover the membrane in transparent plastic wrap.
7. Acquire image using darkroom development techniques for chemiluminescence, or normal image scanning methods for colorimetric detection.

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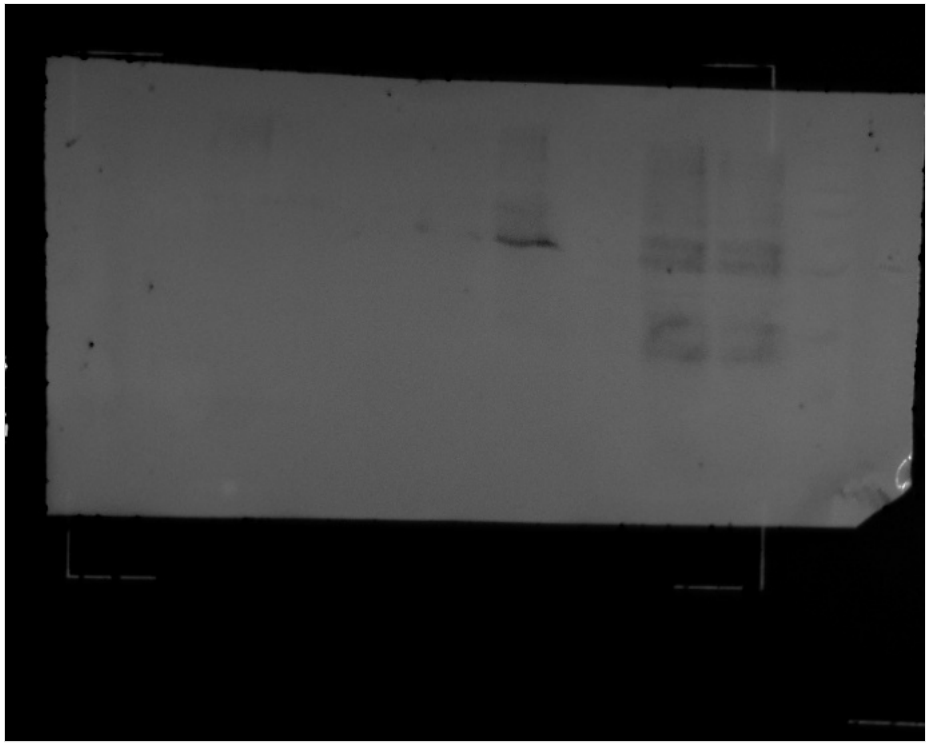
Result:



Date 9.26

CysDes function analysis--acid labile sulfide analysis

Recorder: Shihan Zhu & Wenfei Yu & Meiying Cui

Preparation

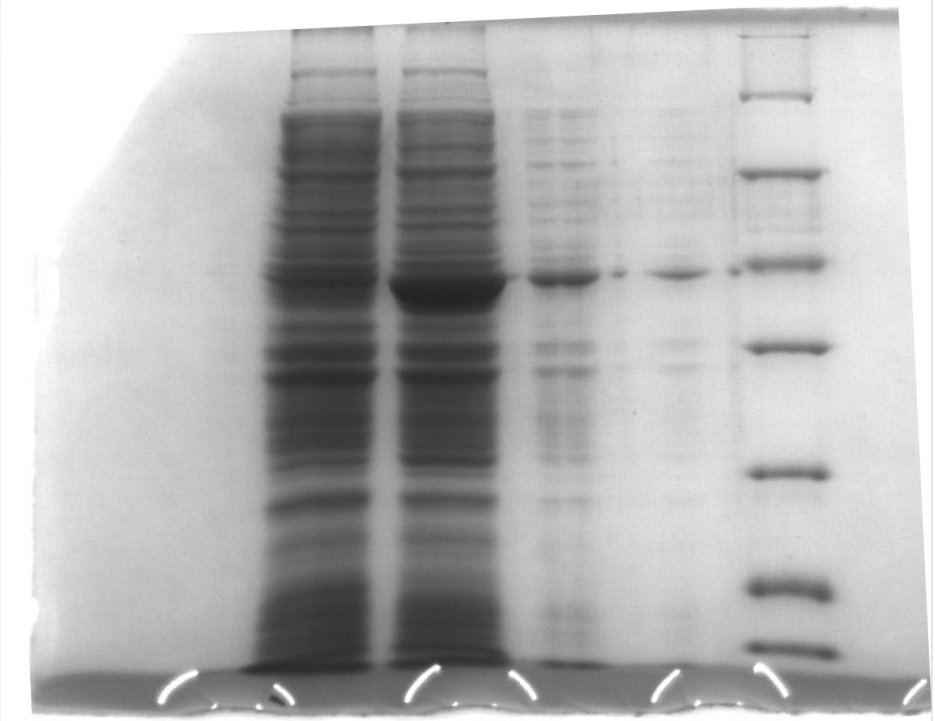
1. Inoculate 2 mL of BL21 with CysDes into 200 mL LB media and add cysteine to final concentration 1 mM and cultivate for about 3 hours, 37°C,250rpm.
2. When OD600 is about 0.4-0.6, add AHL to the media to final concentration 250nM.
3. Cultivate for 3 hours.

Procedure

1. 2 mL of culture samples are centrifuged at 17,000 g for 2 min;
2. The supernatant is removed and the cell pellet is resuspended in 1mL of 0.75 M NaOH;
3. The suspension is then transferred to a EP tube and incubated at 95 degree centigrade for 15 min;
4. Vortex the suspension vigorously and take 25 μL of the suspension to mix with 375 μL of 0.75 M NaOH and 250 μL of 2.6% zinc acetate dihydrate ; 125 μL 0.1% N,N-dimethyl-p-phenylenediamine dihydrochloride in 5 M HCl (freshly prepared) are added to and the solution is vortexes until clear;
5. 50 μL of 11.5 mM FeCl3 in 6M HCl are added and the solution is vortexes and incubated at RT for 30 min;
6. 425 μL of deionized water are added and the OD of the samples are recorded at 670 nm. (Solutions of 0-0.2 mM sodium sulfide in 0.75 M NaOH served as calibration standards.)

SDS-PAGE

帮助



(lane left to right: wt , CysDes-200ul bac/ 30ul , CysDes-166.7ul bac/ 50ul , CysDes-148.1ul bac/ 90ul)

Results

We failed to detect the existence of sulfer ion in our bactria. So we decided to change our protocols for CysDes function analysis.

Date 9.27

PCR of KMADH & pYYDT

Recorder: Xiaoyu Zhang & Menglong Jin

Experimental materials

1. Template: KMADH on pET21 & pYYDT ;
2. Primer: KMADH-r,KMADH-f, pYYDT-f, pYYDT-r. Designed by ourselves, synthesized by General Biosystems;
3. Sterilized ddH2O, 2×PrimeStar(Premix), bought from Takara.

Procedure:

1.Prepare 4 PCR tubes and sequentially add：

sample	**1**	**2**	**3**	**4**
Sterilized ddH2O	22 μL	22 μL	22 μL	22 μL
2×Prime Star(premix)	25 μL	25 μL	25 μL	25 μL
template	1 μL	1 μL	1 μL	1 μL
primer 1	1 μL	1 μL	1 μL	1 μL
primer 2	1 μL	1 μL	1 μL	1 μL
total	50 μL	50 μL	50 μL	50 μL

2.PCR reaction 1,2,3,4 Parameters setting：(for PCR of KMADH)

stage	**temperature**	**time**
step 1	95	10 min
step 2	98	5 s
step 3	56	5 s
step 4	72	1 min

stage	temperature	time
step 5	72	10 min
step 6	4	--

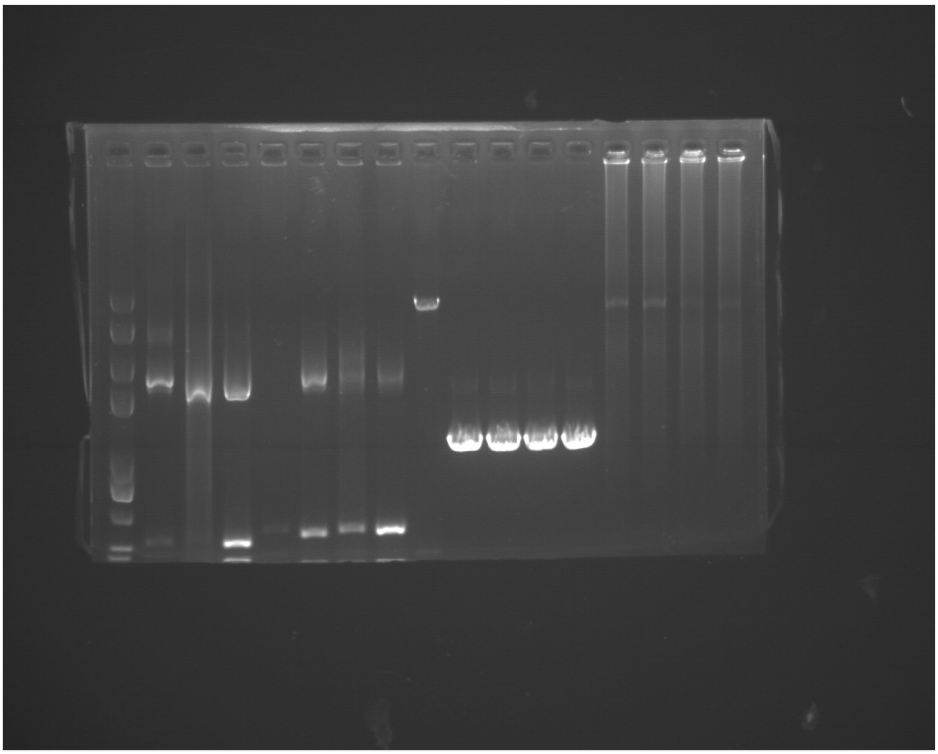
30 cycles(step 2 ~ step 4)

5,6,7,8 Parameters setting：(for PCR of pYYDT)

stage	temperature	time
step 1	95	10 min
step 2	98	5 s
step 3	56	5 s
step 4	72	6 min
step 5	72	10 min
step 6	4	--

30 cycles(step 2 ~ step 4)

Agarose gel electrophoresis Result:



(lane right to left: pYYDT-PCR-1, pYYDT-PCR-2, pYYDT-PCR-3, pYYDT-PCR-4, KMADH-PCR-1, KMADH-PCR-2, KMADH-PCR-3, KMADH-PCR-4)

CysDes function analysis--acid labile sulfide analysis

Recorder: Shihan Zhu & Wenfei Yu & Yonghao Leung

Preparation

1. Inoculate 2 mL of BL21 with CysDes into 200 mL LB media and add cysteine to final concentration 1 mM and cultivate for about 3 hours, 37°C,250rpm.
2. When OD600 is about 0.4-0.6, add AHL to the media to final concentration 250nM.
3. Cultivate for 3 hours and collect the media.
4. Centifuge for 8000rpm,10 min and discard the supernatant.
5. Wash the bacteria with 50 mL TBS and centifuge for 8000rpm,10 min and discard the supernatant.
6. Resuspend the bacteria with 15 mL 1xPBS buffer, and disrupt the cell via ultrasonication(45min, 2s on, 4s off, power level 30%).
7. Centifuge for 14000rpm,20 min and collect the supernatant.

Procedure

sample	0	1	2	3	4	5	6	7	8
Sterilized ddH2O	880 μL	780 μL	680 μL	580 μL	480 μL	780 μL	680 μL	580 μL	480 μL
10x PBS	100 μL	100 μL	100 μL	100 μL	100 μL	100 μL	100 μL	100 μL	100 μL
raw enzyme	0 μL	0 μL	0 μL	0 μL	0 μL	100 μL	200 μL	300 μL	400 μL
wide type extraction	0 μL	100 μL	200 μL	300 μL	400 μL	0 μL	0 μL	0 μL	0 μL
cysteine	20 μL	20 μL	20 μL	20 μL	20 μL	20 μL	20 μL	20 μL	20 μL
total	1 mL	1 mL	1 mL	1 mL	1 mL	1 mL	1 mL	1 mL	1 mL

1. 10 mM PBS (pH 7.5) in a final volume of 1 ml containing 1 or 4 g of purified protein per ml and a 0.5 or 2 mM concentration of the substrates to be analyzed. Add the cysteine, and the mixture was incubated at 37°C for 2 h.
2. Add 0.1 ml of 0.02 M N,N-dimethyl-p-phenylenediamine sulfate in 7.2 N HCl and 0.1 ml of 0.3 M FeCl3 in 1.2 N HCl to the reaction tubes.
3. Inoculate the mixture for 20 min at 20°C.
4. Measure the absorbance at 650 nm.
5. Sulfide concentration was determined from a Na2S standard curve.

Results

(from left to right: sample 0, sample 4, sample 5, sample 8) The sample with our raw enzyme turns green which proves the existence of sulfer ion and the function of CysDes successfully.

Transformation of KMADH into BL21

Recorder: Menglong Jin

Date 9.29

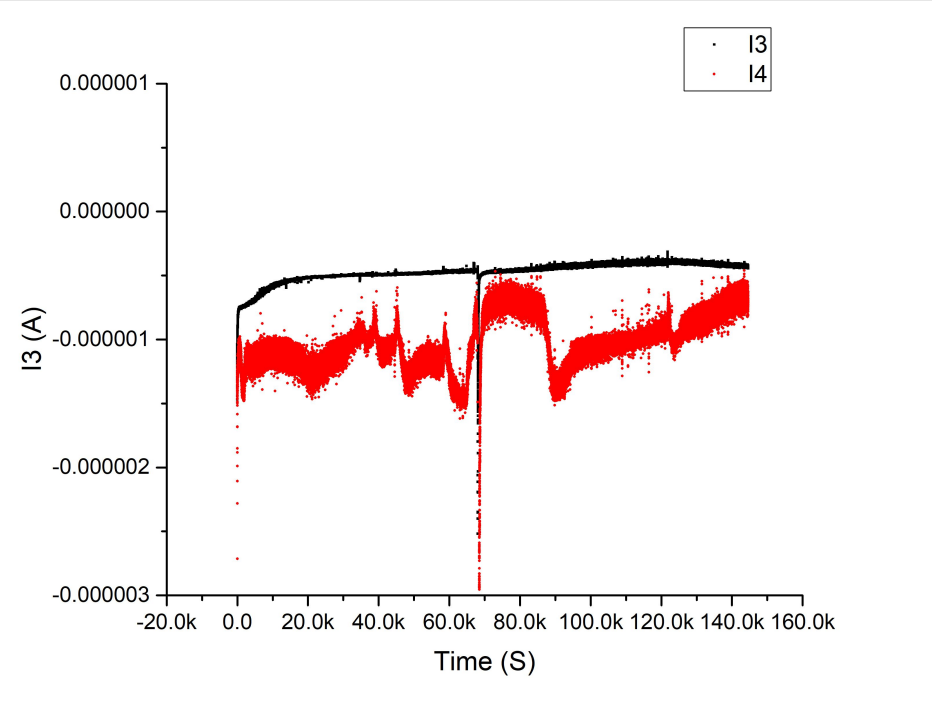
Bio-cathode assay of Mtr

Recorder: Yonghao Liang Strain: WT, Mtr+ccm(induced) Procedure: 1.Prepare the minimal salts medium we need to use:

1. Cultivate the strain overnight in a 200 mL LB medium;
2. Add 1mM IPTG into the culture;
3. Centrifuge half of the final system's volume of the culture at 9000 g, 4℃, 4min and remove the supernatant;
4. Resuspend the bacteria with 20 mL of the minimal salts medium above;
5. Repeat step 4&5 ;
6. Centrifuge the culture at 9000 g, 4℃, 4min, and remove the supernatant;
7. Resuspend the bacteria with 2 mL of minimal salts medium and add them into the system;
8. Run the bio-cathode reaction(-0.5V, sensitivity 10e-4V, total time 9,000,000s, take a sample every second);

9. Add 40 mM of fumaric acid into the system after 24 hours.

Result:



(i4 is the Mtr+ group; i3 is the WT group)

Date 9.30

Preparation of competent cell

Recorder: Yonghao Liang Material: the bacteria that have been co-transformed both Mtr and ccm genes.

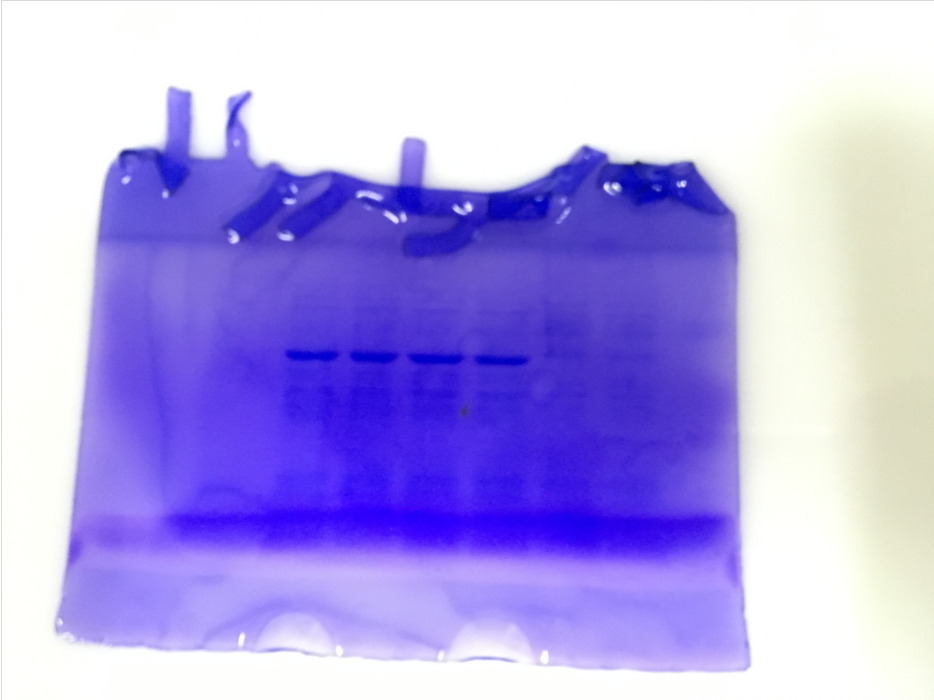
Expression of KMADH

Recorder: Wenfei Yu & Meiying Cui

Material: Strain(KMADH on pET21 in BL21)

Procedure: 1.Inoculate the bacteria from the glycerinum stock and cultivate it overnight, 37℃, 250 rpm; 2.Delute the bacteria into a 5 mL medium and cultivate for 6 hours, 37℃, 250 rpm; 3.Dulute 2 mL of the bacteria above into a 200 mL medium and cultivate it till its OD reach 0.4-0.6; 4.Add appropriate concentration of inducer into the system and induce the expression for 1-3 hours; 5.Take certain volume of bacteria out of the medium, mix with appropriate volume of protein loading buffe, and heat it at 100 ℃ for 20 mins; 6.Use the heated sample for SDS-PAGE.

Result:



帮助

Bio-cathode assay

Recorder: Yonghao Liang Procedure: 1.Prepare the minimal salts medium we need to use:

1. Cultivate the strain overnight in a 200 mL LB medium;
2. Add 1mM IPTG into the induced group;
3. Centrifuge half of the final system's volume of the culture at 9000 g, 4˚C, 4min and remove the supernatant;
4. Resuspend the bacteria with 20 mL of the minimal salts medium above;
5. Repeat step 4&5 twice;
6. Centrifuge the culture at 9000 g, 4˚C, 4min, and remove the supernatant;
7. Resuspend the bacteria with minimal salts medium (the same volume of LB medium we used);
8. Add 18 mM fumaric acid into the system;
9. Cultivate the bacteria at 37˚C, 250rpm, 6h;
10. Centrifuge the culture at 9000 g, 4˚C, 4min, and remove the supernatant;
11. Resuspend the bacteria with 1 mL of minimal salts medium;
12. Pipette the 1 mL of culture onto the graphite electrode and wait till it to dry out;
13. Run the bio-cathode reaction(-0.5V, sensitivity 10e-4V, total time 9,000,000s, take a sample every second);
14. Add 40 mM of fumaric acid into the system and run it for another 12 hours.

Date 10.1

Expression of Mtr

Recorder: Yonghao Liang

material: Strain BL21 that only have Mtr gene but not Ccm A-H gene.

SDS-PAGE Procedure: 1.Inoculate the bacteria from the glycerinum stock and cultivate it overnight, 37˚C, 250 rpm; 2.Delute the bacteria into a 5 mL medium and cultivate for 6 hours, 37˚C, 250 rpm; 3.Dulute 2 mL of the bacteria above into a 200 mL medium and cultivate it till its OD reach 0.4-0.6; 4.Add appropriate concentration of inducer into the system and induce the expression for 1-3 hours; 5.Take certain volume of bacteria out of the medium, mix with appropriate volume of protein loading buffe, and heat it at 100 ˚C for 20 mins; 6.Use the heated sample for SDS-PAGE.

Heme Staining Procedure:

1. Solutions you need for the stain:
- 6.3mM TMBZ in methanol: 60 mg TMBZ in 30mL methanol.
- 0.25M sodium acetate, pH 5.0
- 30% hydrogen peroxide (9.79M, in the fridge)
- Isopropanol
2. Make the TMBZ solution while the gel is running. This takes time to solubilize. Co
3. Rinse the gel with water for 5 minutes
4. Immediately before use, mix 30mL TMBZ solution with 70mL sodium acetate solution (
5. Immerse the gel in the solution above. put gel in the dark. (ie a drawer)
6. Mix occasionally (every 15 minutes) for 1-2 hours
7. Add 495 µL 30% hydrogen peroxide (final concentration 30 mM in 100 mL). Mix well.
8. Staining should be visible within 3 minutes and increases in intensity over the ne
9. Background of the gel may be removed by destaining with 3:7 isopropanol:0.25M sodi

Result: SDS-PAGE:

Heme-staining:

Transformation of KMADH into co-transformed competent cell

Recorder: Yonghao Liang

NOTE:Generally, competent bacteria are restrored in -70 degree centrigrade environment. 1.Take the competent bacteria from refrigerator and incubate them into ice about 5 mins until it is dissolved 2.Absorb 10µL Mtr-pET28 and mix it wµith bacteria solution thoroughly. ATTENTION: Please operate this step tenderly!!! 3.Put the tubes on the ice about 30 mins.(Time SHOULD BE ACCURATE) 4.Make a heat shock at 42 degree centigrade about 90 sec(TIME SHOULD BE ACCURATE) 5.Put the tubes on the ice about 5 mins again. 6.Add 900 µL LB medium into EP tubes and cultivate the bacteria at 37 degree centrigrade about 60 min. 7.Centrifuge them at 4000 rpm for 2 minutes and we will see sediment in the tubes. 8.Discard the supernatant liquid and leave about 200 µL medium. 9.Coat plate: Add 200 µL solution in a plate with kanamycin. 10.Cultivate these bacteria overnight for further use.

Date 10.2

Growth curve of bac that contain CysDes under different concentration of Cd2+

Recorder: Yonghao Liang

Growth curve of bac that contain KMADH under different concentration of alcohol and ethanal

Recorder: Yonghao Liang

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