**Cloning Mta-Map operon from *M. stadtmaneae* into *M. maripaludis* (April 28, 2015)**

**Objective:** The goal of this cloning is to create a new strain of *M. maripaludis* that has the 5 gene operon from *M. staudmanii* that codes for methanol methyltransferase (mta) and methytransferase activating protein (map). What’s novel here is that we’re using the smaller plasmid (pAW42C) to put in these genes because we think we’ll have better success that when we used the larger plasmid to attempt this.

**Basic Procedure:** We start with our 5 gene operon (our insert) in pUC57Kan, a common *E. coli* cloning vector. We want to make lots of our insert and keep the bookending *Nsi*I and *Bgl*II restriction sites intact, so we run a PCR with primers that amplify the sequence between these two sites, then run the results in a gel to confirm what they are. Once we’ve confirmed, we digest it and pAW42C, then ligate it in there and transform the whole mess into *E. coli.* We plate that into selective media to select for only bugs with our operon and take single colonies that we can test for the insert. We save some of each colony in media, then boil the other one to lyse the cells and screen by PCR with screening primers. For candidates that look right (vector + insert are in there), we use the saved bits from media, grow them up overnight, then purify the plasmid using a kit. We digest the plasmids we get to make sure it matches what it should, and if it does then we sequence it, grow more cells with it to make more, and put it in the strain collection. Perhaps most importantly, we then use the grown up plasmid to transform *M. maripaludis* and then test for expression using a Western and an RT-PCR.

**Specific Procedure:**

# 04.28.2015 Design and order primers

Fwd: 5’-CCGAAAGACACAAATATATAGAGGCCTAATGCATGG-3’ (EGP57)

Rev: 5’-GGATAACAATTTCACACAGGAAACAGCTATGACCAT-3’ (EGP58)

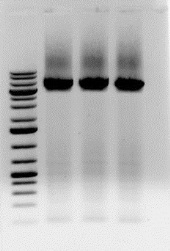
\*Note: Fwd was incorrectly copied over on order; it was made as described above, but it should have been: 5’-CCGAAAGACAACAATATATAGAGGCCTAATGCATGG-3’

# 04.29.2015 Amplify the operon using PCR

First run is with 3 different annealing temps (53, 55, 57) to see what works best for selectivity. Solutions were made as follows:

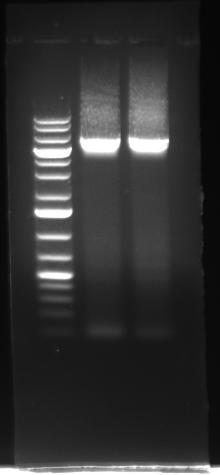
|  |  |  |
| --- | --- | --- |
| Reagent | Volume (μL) | 3.5x Volume (μL) |
| Water | 33.5 | 117.25 |
| 5X iProof HF Buffer | 10.0 | 35 |
| 10mM dNTPs | 1.0 | 3.5 |
| Fwd primer | 1.0 | 3.5 |
| Rev primer | 1.0 | 3.5 |
| Template | 1.0 | 3.5 |
| iProof polymerase | 0.5 | 1.75 |
| Total Volume: | 50.0 | 175.0 |

# 04.30.2015 Amplify the operon using PCR (attempt #2)

First run gave poor results (no gel image available because it was tossed). It became apparent that something was missing or forgotten in the first run, so we repeated the experiment. Second run used same exact solutions and temperatures, but Eli performed it. Gel image on right confirmed that we amplified the insert successfully. Solutions as follows:

|  |  |  |
| --- | --- | --- |
| Reagent | Volume (μL) | 3.5x Volume (μL) |
| Water | 33.5 | 117.25 |
| 5X iProof HF Buffer | 10.0 | 35 |
| 10mM dNTPs | 1.0 | 3.5 |
| Fwd primer | 1.0 | 3.5 |
| Rev primer | 1.0 | 3.5 |
| Template | 1.0 | 3.5 |
| iProof polymerase | 0.5 | 1.75 |
| Total Volume: | 50.0 | 175.0 |

# 05.01.2015 Amplify the operon using PCR (attempt #3)



Following Eli’s successful run, I performed the third run at 57C as annealing temp, because higher is generally better if all else is equal. We were concerned that we would probably have to gel purify based on the streakiness Eli got, so I made a larger master mix with 4 tubes instead of 3.

|  |  |  |
| --- | --- | --- |
| Reagent | Volume (μL) | 4.5x Volume (μL) |
| Water | 33.5 | 150.75 |
| 5X iProof HF Buffer | 10.0 | 45 |
| 10mM dNTPs | 1.0 | 4.5 |
| Fwd primer | 1.0 | 4.5 |
| Rev primer | 1.0 | 4.5 |
| Template | 1.0 | 4.5 |
| iProof polymerase | 0.5 | 2.25 |
| Total Volume: | 50.0 | 225.0 |

Results shown on right indicated that our insert DNA is not particularly pure, so we need to gel purify.

# (DATE MISSING) Gel purify the DNA

* 1. Empty tube weight: 6.646
  2. Full tube weight: 7.189
  3. DNA gel weight: 0.543 g (543 mg)

\*\*\*\*\*\*\*

MISSING HERE:

I’m missing dates and images for this at the moment and will have to fill in the gaps, but I’m fairly certain one of us has it written somewhere. Essentially, here’s what’s missing:

-Gel purification resulted in a tiny amount of DNA. So I re-ran with a huge well and something like 8 reactions worth of materials.

-Re-run was again gel purified, but we once again got a tiny amount of DNA. We decided to skip the purification next time and just do one of the kits (I forget what it’s called…maybe QIAquick?)

-I re-ran with 8 reactions again I believe, and this time the DNA got prepped and used immediately on the following steps

From Eli:

While Matt’s larking off in Denmark, I will try to carry his cloning forward.

# 5.15.15 Onward and Upward – Cut and SAP

He has amplified and purified the Methanosphaera stadmanae artificial mta operon. The product is 5,976bp and his concentrations are 877ng/μL (tube 1 of 2) and 887ng/μL (tube 2 of 2).

I’ll supply the vector, pAW42C (prep 3 of 3 from 4.28.15), which is 4,934bp and has a concentration of 1,247ng/μL.

**Molar-ends calculations (#’s in red are specific to the DNA in question)**

μg DNA x 1pmol x 106pg x 1 x 2 x 1 kb

660pg μg N 1000bp

* W
* 660 pg/pmol is the average molecular weight of a single nucleotide pair
* 2 is the number of ends in a linear DNA molecule
* kb/1000bp is a conversion factor for kilobases to base pairs

**Insert Ends Calculation**

877μg/mL x 1/660 x 106 x 1/5.98 x 2 x 1/1000

= **444 pmoles of ends / 1μL of purified DNA** [this calculation was for prep 1 of 2, shown above]

**Vector Ends Calculation**

**1,247μg/mL x 1/660 x** 106 x 1/4.93 x 2 x 1/1000

= **766 pmoles of ends / 1μL of purified DNA**

Cut with *Nsi*I and *Bgl*II:

DNA Species

Reagent Vector Insert

water 36.5 34.5

\*of a 1:10 dilution of the vector prep.

10X Buffer 3.1 4.5 4.5

DNA prep 1.0\* 3.0

*Nsi*I 1.5 1.5

*Bgl*II 1.5 1.5

Total volume 45.0μL 45.0μL

total pmoles ends 76.6 1,332

pmoles ends/μL 1.70 29.6

Incubated 1 hour at 37ºC, then accidentally added 56μL of sterile water to the vector digest rather than transferring half the vector digest to a fresh tube and THEN adding the water. So the new numbers for the digests are:

Reagent Vector Insert

Total volume 101.0μL 45.0μL

total pmoles ends 76.6 1,332

pmoles ends/μL 0.758 29.6

With a few recalculations, I carried on as follows: I transferred 23μL of the vector reaction to a fresh tube, then add the following reagents to the transferred half of the vector reaction:

Reagent Vector Insert

*Nsi*I/*Bgl*II rxn 23.0

water 103.5 --.--

10X Buffer 3.1 26.5 --.--

rSAP 17.0 --.--

New Total volume 170.0μL 45.0μL

total pmoles ends 17.4 1,332

**pmoles ends/μL 0.102 29.6**

Incubated a further 30’ at 37ºC.

Inactivated enzymes at 80ºC for 20’. Stored at -20ºC overnight.

# 5.21.15 Ligation and Transformation

Vector : Insert ratio 🠂 1:1 1:29

Water 9.0 9.0

10X Fast-Link Buffer 1.5 1.5

10mM ATP 1.5 1.5

vector DNA 1.0 = 0.102pmole ends 1.0 = 0.102 pmole ends

insert DNA 1.0\* = 0.102pmole ends 1.0\*\* = 2.96 pmole ends

Fast-Link Ligase 1.0 1.0

Total volume (μL) 15.0μL 15.0μL

\*of a 1:290 dilution \*\*of a 1:10 dilution

Incubated 20’ on bench then inactivated ligase 15’ at 75ºC.

Transformed 2μL of each ligation reaction into 70μL of XL10 Gold competent cells. Plated 50μL of each recovery culture on LAamp plates.

# 5.22.15 Screening PC reactions

Only 5 colonies, all about the same size, grew on the 1:1 plating, while the 1:20 plate had 11 colonies, most were smallish. there were two to three colonies in the larger size range and these were about the same size as those on the 1:1 plate. I concentrated and plated the remainder of each recovery culture and will screen those on Sunday, if need be. Judging from past experience with these operon insertions, it will be necessary.

In the meantime, I ran pc reactions to screen the 16 candidates I had in hand, plus a vector control:

Volume (µL)

Reagent 1 rxn 19 rxns

water 31.5 598.5

5X iProof HF Buffer 10.0 190.0

10mM dNTP’s 1.0 19.0

fwd primer\* 1.0 19.0

rev primer\*\* 1.0 19.0

template\*\*\* 5.0 --.---

iProof polymerase 0.5 9.5

Total volume 50.0

\* Forward primer = EGP55.

\*\* Reverse primer = EGP56.

\*\*\* Boil-preps of candidate colonies; the no-insert control was pJAR50C

Thermalcycler program:

Cycle Temperature Time # Cycles

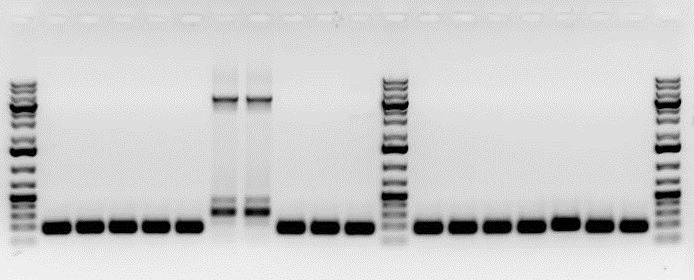
initial denaturation 98ºC 1’ 1

denaturation 98ºC 10”

annealing **53ºC** 10” 35

extension 72ºC 2’

final extension 72ºC 1’ 1



Ladder:

20,000

10,000

7,000

**5,000**

4,000

3,000

2,000

**1,500**

1,000

700

**500**

400

300

200

75

1. GeneRuler 1kb Plus
2. candidate #1
3. #2
4. #3
5. #4
6. #5
7. **#21**
8. **#22**
9. #23
10. #24
11. pJAR50C control
12. GeneRuler 1kb Plus
13. #25
14. #26
15. #27
16. #28
17. #29
18. #30
19. #31
20. GeneRuler 1kb Plus

Streaked candidates 21 and 22 on LAamp for colony purification.

I’ve returned from “larking” and started doing things again here

# 5.26.15 Beginning subclone into pJAR50CT

Will use the same stock of cut insert DNA as above and simply cut the new vector. The vector used here was purified on 5.24.15 (prep 1 of 3; isolate 2M), and this prep had a concentration of 1,380μg/mL. The vector is 5078bp.

**Vector Ends Calculation**

**1,380μg/mL x 1/660 x** 106 x 1/5.08 x 2 x 1/1000

= **823 pmoles of ends / 1μL of purified DNA**

Cut pJAR50CT vector with *Nsi*I and *Bgl*II:

Reagent Volume

water 40.0

\*of a 1:10 dilution of the vector prep.

10X Buffer 3.1 5.0

DNA prep 1.0\*

*Nsi*I 2.0

*Bgl*II 2.0

Total volume 50.0μL

total pmoles ends 82

pmoles ends/μL 1.6

Reagent Vector Insert ‡

‡ Same prep as that on page 4 above. No further manipulations performed here.

*Nsi*I/*Bgl*II rxn 10.0

water 118.0 --.--

10X Buffer 3.1 16.0 --.--

rSAP 16.0 --.--

New Total volume 160.0μL 45.0μL

total pmoles ends 16 1,332

**pmoles ends/μL 0.1 29.6**

Incubated a further 30’ at 37ºC.

Inactivated enzymes at 80ºC for 20’. Stored at -20ºC overnight.

# 5.26.15 Ligation and Transformation

Vector : Insert ratio 🠂 1:29

Water 9.0

10X Fast-Link Buffer 1.5

10mM ATP 1.5

vector DNA 1.0 = 0.100pmole ends

insert DNA 1.0\* = 2.96pmole ends

Fast-Link Ligase 1.0

Total volume (μL) 15.0μL

\*of a 1:10 dilution

Incubated 20’ on bench then inactivated ligase 15’ at 75ºC.

Transformed 2μL of each ligation reaction into 70μL of XL10 Gold competent cells. Plated 50μL and, separately, the remainder of each recovery culture on LAamp plates.

# 5.28.15 Repeat Ligation and Transformation for pJAR50CT

Eli did the previous step and only got 2 cultures to screen. She reserved the plates and I repeated the ligation with exactly the same materials, with a small change in the amounts:

Vector : Insert ratio 🠂 1:29

Water 5.0

10X Fast-Link Buffer 1.5

10mM ATP 1.5

vector DNA 3.0 = 0.300 pmole ends

insert DNA 3.0\* = 8.88 pmole ends

Fast-Link Ligase 1.0

Total volume (μL) 15.0μL

\*of a 1:10 dilution

Incubated 20’ on bench then inactivated ligase 15’ at 75ºC.

Transformed 2μL of each ligation reaction into 70μL of XL10 Gold competent cells. Plated ~500 μL of each recovery culture on an LB-amp plate.

# 5.29.15 Screening PC reactions

We only had 4 colonies total between my plates and Eli’s, so I did the boil prep on all of these and setup the following reactions:

Volume (µL)

Reagent 1 rxn 6 rxns

water 31.5 186

5X iProof HF Buffer 10.0 60.0

10mM dNTP’s 1.0 6.0

fwd primer\* 1.0 6.0

rev primer\*\* 1.0 6.0

template\*\*\* 5.0 --.---

iProof polymerase 0.5 3.0

Total volume 50.0

\* Forward primer = EGP55.

\*\* Reverse primer = EGP56.

\*\*\* Boil-preps of candidate colonies; the no-insert control was pJAR50CT

Thermalcycler program:

Cycle Temperature Time # Cycles

initial denaturation 98ºC 1’ 1

denaturation 98ºC 10”

annealing **53ºC** 10” 35

extension 72ºC 2’

final extension 72ºC 1’ 1

Following completion of the PCR, we stored the results in the freezer over the weekend to await gel imaging. Gel was imaged on 6/01/2015 (awaiting pic, which was on Dinky-Doo). The gel showed clearly that we didn’t get the construct we were looking for (our operon insert inside of pJAR50CT). So we’ll try again.

# 6.8.2015 Ligation of pJARCT: Attempt 3

# 6.9.2015 Picking Colonies, Screening PCR, and Imaging

Ran PCR results in 4 gels on 6/10/2015 but got no viable candidates.

# 6.11.2015 Re-Cutting, Ligating, and Transforming (Attempt 4)

I didn’t get any candidates yesterday on my 4 gels, so now we’re going back to the cutting and making sure we do that correctly, then do the next steps. Starting with uncut pJAR50CT and insert (“Nancy”; I’m Nancy 1 and she’s Nancy 2), cutting them, ligating them, transforming E. coli, and plating. First up is cutting, including rSAP-ing:

(ELI’S table should be copied here)

To check the cutting, I ran a gel with both her and my attempts, because we’re doing this side by side. Gel image and lane description follows:

Ligation mixture (based on previous experience):

Vector : Insert ratio 🠂 1:20 (C) 1:20 (D)

Water 8.0 2.0

10X Fast-Link Buffer 1.5 1.5

10mM ATP 1.5 1.5

vector DNA 1.0 = 0.1 pmole ends 3.0 = 0.3 pmole ends

insert DNA 2.0\* = 2.0 pmole ends 6.0\* = 6.0 pmole ends

Fast-Link Ligase 1.0 1.0

Total volume (μL) 15.0μL 15.0μL

\*of a 1:10 dilution

Incubated 20’ on bench then inactivated ligase 15’ at 75ºC.

Froze ligation results for transformation the following week

# 6.11.2015 Ligating and Transforming (Attempt 4 & 5)

Transformed ligations (C and D) from 6/11 into XL-10 ultra competent cells and streaked. Eli’s parallel ligations didn’t turn out well, so we’re trying some different ligation ratios as well. Ligation mixtures are as follows:

Vector : Insert ratio 🠂 1:10 (E) 1:3 (F)

Water 7.0 3.2

10X Fast-Link Buffer 1.5 1.5

10mM ATP 1.5 1.5

vector DNA 2.0 = 0.2 pmole ends 6.0 = 0.6 pmole ends

insert DNA 2.0\* = 2.0 pmole ends 1.8\* = 1.8 pmole ends

Fast-Link Ligase 1.0 1.0

Total volume (μL) 15.0μL 15.0μL

\*of a 1:10 dilution

Incubated 20’ on bench then inactivated ligase 15’ at 75ºC.

Transformed into XL-10 ultra competent cells and plated for colonies. Will select them tomorrow