Week 8: 27. July 2015- 31. July 2015

27. July 2015

- The sequencing revealed a point mutation in the pET-30 vector backbone when we clone *medh2*. Therefore we decided to clone *medh2* into a pET-30 isolated from a different clone. For our other pET-30 construct the mutation is not significant because it is in the region we cut out.

1) Invitro mutagenesis of *hps* to remove PstI Restriction Site for BioBrick System
   - Use QuickChange Site-Directed Mutagenesis Kit (Agilent) and follow the provided manual
   - Primer: hps_mut3/mut4
   - Template: pCR4+ *hps*-mut XbaI

   a) DpnI Treatment
   - Add 1 µl of DpnI to the PCR Reaction and incubate at 37 °C for 1 hour

   b) Transformation of *E. coli* TOP10 cells with DpnI treated PCR reaction
   - Add 1 µl of DpnI treated PCR reaction to chemically competent *E. coli* TOP10 cells.
   - Following the protocol for transformation of chemical competent *E. coli* cells
   - Plate on LB+Amp [100 µg/ml] and incubate overnight at 37 °C

2) Digest of pET-30 with EcoRI/ XhoI
   - Pipetting scheme according to the protocol Restriction Digest
   - Adding directly both enzymes and incubate the Reaction at 37 °C for 1 hour.
   - Verify 10 ul of the digestion on agarose gel

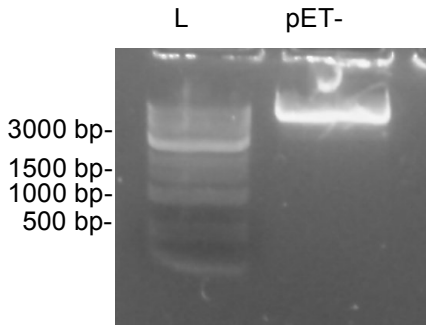

Figure 1: Digest of pET-3*0* with EcoRI/ XhoI 10 µl of Digest were checked on 1% (w/v) agarose gel. Expected sizes: pET-30- 5388 bp. As ladder (L) 1kB Ladder (NEB) was used.

1) Purification of digested pET-30
   - Using Wizard® SV Gel and PCR Clean-Up System (Promega) and following the provided manual
2) Dephosphorylation of opened pET-30 using shrimp alkaline phosphatase
   - Pipetting scheme according to the protocol Shrimp Alkaline Phosphatase Treatment (Fermentas).

3) Ligation of *medh2* into pET-30
   - Pipetting scheme according to the Protocol Ligation with T4 DNA Ligase (NEB)
   - Use dephosphorylated pET-30
   - Control Reaction: pET-30 Selfligation
   - Incubate the reaction at 16 °C overnight
4) Inoculation of preculture culture for pilotexpression of MMOB, MMOC, MMOD, MMOG in *E. coli* BL21
   - Inoculate one clone with 5 ml LB+Kan [50 µg/ml] and incubate overnight at 37 °C shaking at 220 rpm.
   - As negative control inoculate a 5 ml preculture of *E. coli* BL21 cells containing the selfligated pET-30

28. July 2015

Transformation of *E. coli* TOP10 cells with pET-30+*medh2* and pET-30 selflligated

- Add 5 µl of Ligation reaction to chemically competent *E. coli* TOP10 cells.
- Following the Protocol for Transformation of chemical competent *E. coli* cells
- Plate on LB+Kan [50 µg/ml] and incubate overnight at 37 °C

Pilotexpression of MMOB, MMOC, MMOD, MMOG

- Measure OD_{600} of each preculture
- Inoculate an 5 ml expression culture (LB+Kan [50 µg/ml]) with an OD_{600} of 0.3
- Grow the expression culture for 1 hour at 37 °C shaking at 220 rpm.
- Measure OD_{600} again
- Take a 1 ml sample in an uninduced stage and harvest the cell by centrifugation at 8,000 x g for 5 minutes
- Induce protein expression by adding IPTG (Final Concentration: 1mM).
- Incubate the culture for 3 hours at 37 °C shaking at 220 rpm.

- Take 1 ml sample 3 hours after induction and harvest the cells by centrifugation at 8,000 x g for 5 minutes
- Keep the cell pellets at 4 °C
- Treat the negative control in the same way!

5) Inoculation of liquid culture for plasmid isolation of pCR4+*hps*-mut XbaI/PstI
   - Inoculate one clone with 5 ml LB+Amp [100 µg/ml] and incubate overnight at 37 °C shaking at 220 rpm

6) Inoculation of preculture culture for solubility assay of MMOX, MMOY, MMOZ,
   - Inoculate one clone with 5 ml LB+Kan [50 µg/ml] and incubate overnight at 37 °C shaking at 220 rpm.
   - As negative control inoculate a 5 ml preculture of *E. coli* BL21 cells containing the selfligated pET-30

29. July 2015

1) SDS-PAGE and Coomassie staining to verify successful pilotexpression of MMOB, MMOC, MMOD, MMOG
   - Prepare Samples for SDS-Page:
     - Add 100 µl 1x SDS-Loading Buffer
     - Boil the sample at 95 °C for 20 minutes
   - Run SDS-Page
     - 18 % Seperating Gel
     - load 10 µl
   - Stain for 1 hour in Coomassie Staining Solution
   - Destain overnight

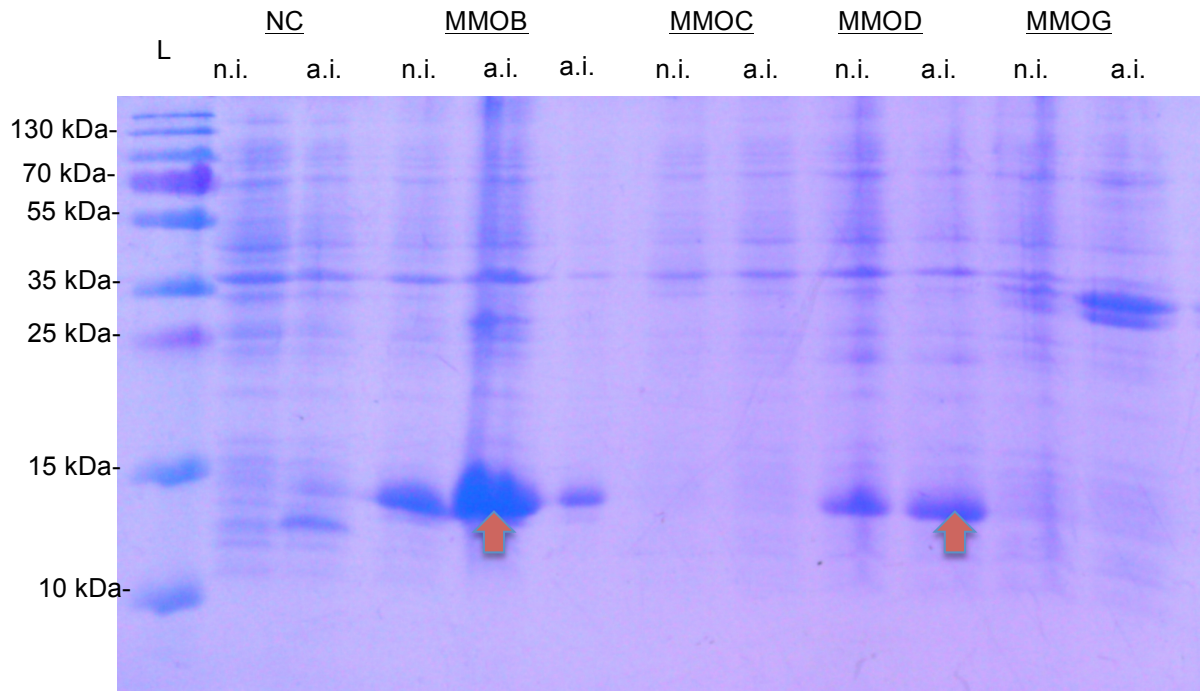

Figure 2: Pilotexpression of MMOB, MMOC, MMOD, MMOG in *E. coli* BL21. Coomassie staining of heterologously expressed MMOB, MMOC, MMOD, MMOG in pET-30. 1 ml samples were taken in an uninduced stage (n.i.) and 3 hours after induction (a.i.) with 1 mM IPTG. Theoretical weights: MMOB-15.95 kDa, MMOC-38.48 kDa, MMOD-11.91 kDa, MMOG-59.38 kDa. Orange arrows determine the appropiate protein. As ladder PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa (ThermoScientific) was used. As negative control (NC) selfligated pET-30 was used for expression in *E. coli* BL21. Proteins were separated by SDS-PAGE.

2) Expression of MMOX, MMOY, MMOZ for solubility assay

- Measure OD_{600} of each preculture
- Inoculate an 5 ml expression culture (LB+Kan [50 µg/ml]) with an OD_{600} of 0.3
- Grow the expression culture for 1 hour at 37 °C shaking at 220 rpm.
- Measure OD_{600} again
- Take a 1 ml sample in an uninduced stage and harvest the cell by centrifugation at 8,000 x g for 5 minutes
- Induce protein expression by adding IPTG (Final Concentration: 1mM).
- Incubate the culture for 3 hours at 37 °C shaking at 220 rpm.
- Take 1 ml sample 3 hours after induction and harvest the cells by centrifugation at 8,000 x g for 5 minutes
- Keep the cell pellets at 4 °C

3) Plasmid Isolation of pCR4+*hps*-mut XbaI/PstI

- Use the QIAprep Spin Miniprep Kit (Qiagen) and follow the provided manual.
- Send for Sequencing
    - Sequencing revealed a succesful mutagenesis

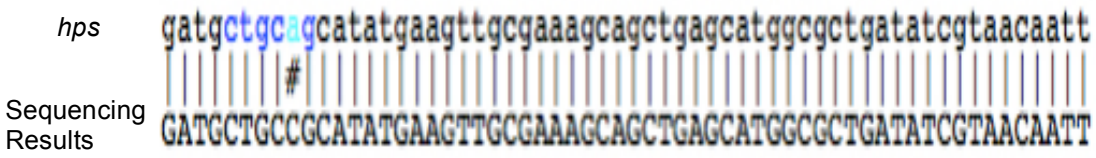

Figure 3: Alignment of *hps* original sequence with sequencing results of *hps* invitro mutagensis to remove PstI restriction site. Blue colour indicates the PstI restriction site in the *hps* sequence. Cyan labeled A in the *hps* sequence indicates the base we wanted to change into a C.

4) Colony-PCR to screen for clones containing the pET-30+*medh2* construct

- Constructs that needed to be identified: pET-30+*medh2*
- Pipetting scheme and PCR program according to PCR with Taq-DNA Polymerase Protocol
- Primer: medh2_E1/E2
- Check 6 Clones
- Positive Control: add 1 µl of pCR4+medh2
- Negative Control: add 1 µl MilliQ Water

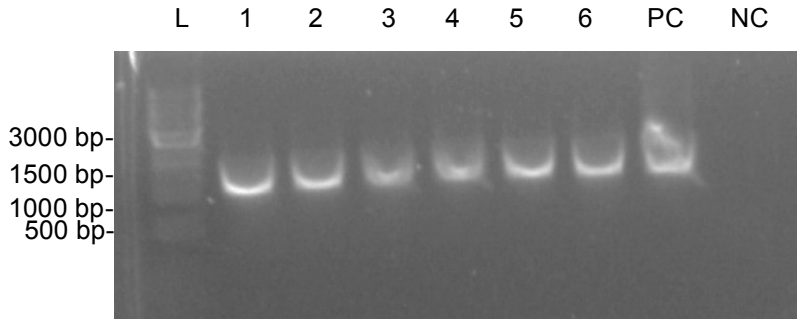

Figure 4: Colony-PCR to identify clones containing pET-30+*medh2* expression construct.
PCR with Taq-DNA Polymerase to identify pET-30+*medh2*. Numbers 1-6 determine the checked clone. As positive control (PC) 1 µl pCR4+*medh2* was used. As negative control (NC) 1 µl MilliQ Water was added. 10 µl of PCR were analzyed on 1 % (w/v) agarose gel. Expected sizes: *medh2*-1208 bp. As ladder (L) 1 kB Ladder (NEB) was used.

5) Inoculation of liquid culture for plasmid isolation of pET-30+*medh2*

- Inoculate clone with 5 ml LB+Kan [50 µg/ml] and incubate overnight at 37 °C shaking at 220 rpm.

30. July 2015

Solubility Assay, SDS-PAGE and Coomassie staining to verify soluble expression of MMOX, MMOY, MMOZ

- Solubility Assay
  - Resuspend the cell pellet in 600 µl PBS buffer
  - Add glas beads
  - Use machine to disrupt the cells
  - Centrifuge at 16,000 x g for 5 minutes
  - Transfer 600 µl supernatant to a new tube—> soluble fraction
  - Resuspend the pellet in 300 µl PBS buffer → Insoluble fraction
- Prepare Samples for SDS-Page:
  - Add 200 µl 4x SDS-Loading Buffer
  - Boil the sample at 95 °C for 20 minutes
- Run SDS-Page
  - 18 % Seperating Gel
  - load 10 µl
- Stain for 1 hour in Coomassie Staining Solution
- Destain overnight

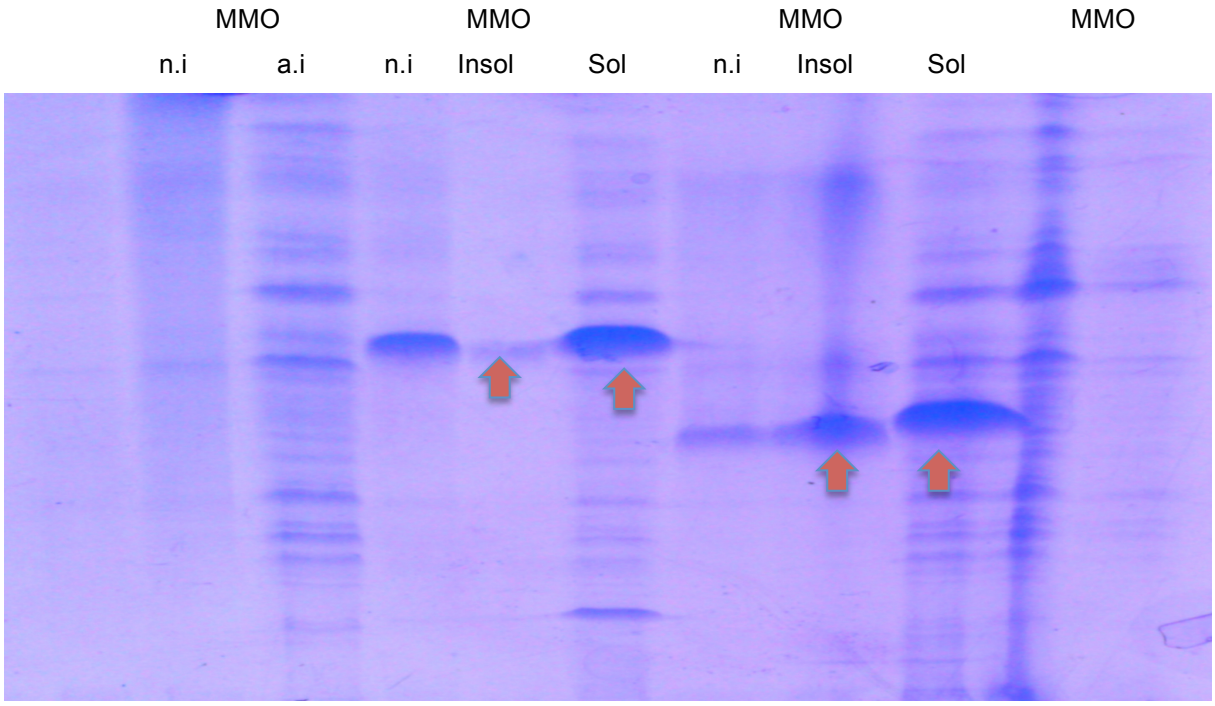

Figure 5: Solubility assay MMOX MMOY MMOZ. Coomassie staining of solubility assay of MMOX, MMOY, MMOZ. Proteinexpression was performed for 3 hours. Cell pellet was lysed and separated into soluble (sol) and insoluble (insol) Fraction. As control 1 ml sample was taken in an uninduced stage. Theorteical molecular weight:

MMOX-60.56 kDa, MMOY-45.06 kDa, MMOZ-19.81 kDa. Expression of MMOC was rechecked from 29.07.2015. Proteins were separated by SDS-PAGE.

Plasmid Isolation of pET-30+*medh2*

- Use the QIAprep Spin Miniprep Kit (Qiagen) and follow the provided manual.
- Send the plasmids for sequencing with T7-FWD primer
    - Sequencing revealed a point mutation in the vector backbone. Same point mutation as at (date)
    - We identified the used vector as pET-30b, not as previously assumed pET-30a

Colony-PCR to screen *E. coli* BL21 clones for presence of the pET-30+*mmoG* and pET-30+*mmoC* construct

- Since the protein expression of MMOG and MMOC failed 2 times we wanted to verify that our expression strain contains the right construct
- Constructs: pET-30+*mmoG*, pET-30+*mmoC*
- Pipetting scheme and PCR program according to PCR with Taq-DNA Polymerase Protocol
- Primer: mmoG_E1/E2, mmoC_E1/E2
- Check 6 Clones
- Positive Control: add 1 µl of pET30+*mmoC* and pET-30+*mmoG*
- Negative Control: add 1 µl MilliQ Water

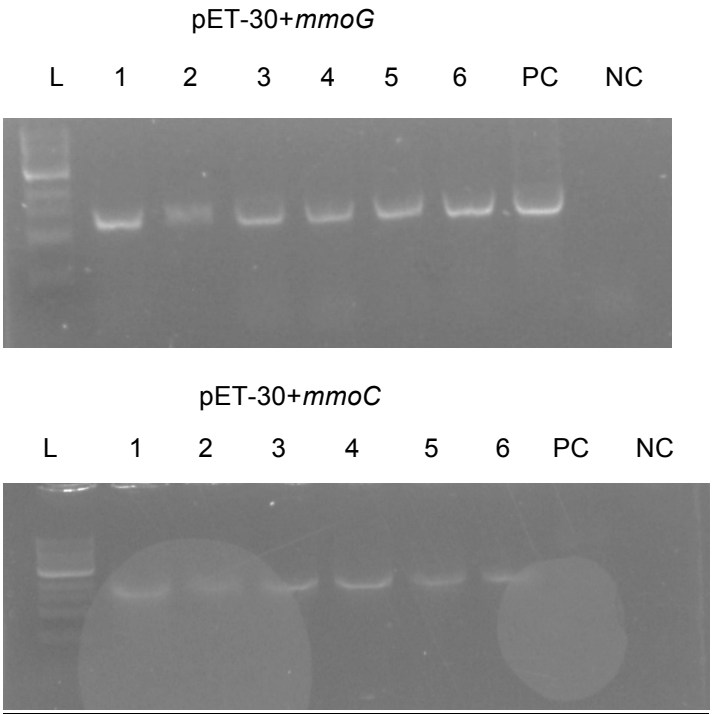

Figure 6: Colony-PCR to identify clones containing pET-30+*mmoG* and pET-30+*mmoC* expression construct. PCR with Taq-DNA Polymerase to identify pET-30+*mmoG* and pET-30+*mmoC*. Numbers 1-6

determine the checked clone. As positive control (PC) 1 µl pCR4+*medh2* was used. As negative control (NC) 1 µl MilliQ Water was added. 10 µl of PCR were analzyed on 1 % (w/v) agarose gel. Expected sizes: *mmoC*-1088 bp *mmoG*-1708 bp. As ladder (L) 1 kB Ladder (NEB) was used.

6) Sending pET-30+*mmoG* for sequencing

- Sequencing revealed 20 bp deletion in the middle of *mmoG*→ pET-30+*mmoG* construct is not correct, which might explain the problems with successful protein expression of MMOG