QGEM Laboratory Notes

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Project: Biliverdin Production via HO-1 catalyzed breakdown of Heme

June 8, 2009

- Requested sequence of I716390 from the Berkeley 2007 iGEM team (Bactoblood)

June 17, 2009

- Ordered agar stabs of I716152 (hemA), I716153 (hemB), and I716390 (heme composite) transformed into Top10 from iGEM HQ

June 26, 2009

- Received glycerol stocks of I716154 (hemC) and I716155 (hemD) transformed into Top10 from Laboratory 1 – these were included in the 2009 distribution

July 6, 2009

- Five test tubes containing 100 μg/mL of ampicillin in 5mL of LB were inoculated with I716152, I716153, I716154, I716155, and I716390 and left in a spinning rack overnight at 37°C

July 7, 2009

- The five overnight cultures from July 6 were miniprepped using a Genelute Plasmid Miniprep Kit

July 8, 2009

- Received rehydrated DNA stocks of pSB1A2 and pSB4C5, and miniprepped plasmids K098010 from Laboratory 1 – these were included in the 2009 distribution

- In order to verify the identity of the miniprepped plasmids, a one-hour EcoRI digestion of I716152, I716153, I716154, I716155, I716390, and K098010 was performed

- 1mL of the I716390 overnight culture was pelleted and the supernatant was analyzed with a wavelength scan in the spectrometer (blanked with LB), which showed a single peak at 407.8nm (according to the Bactoblood wiki and Moffet *et al* 2002., heme should peak at 412nm)

July 9, 2009

- The EcoRI-digested plasmids from July 8 were run on a 0.8% agarose gel with negative controls of undigested plasmids

- I716152, I716153, and I716154 were run on one gel and I716155, I716390, and K098010 were run on another
- Both gels were unsuccessful in that we did not obtain the correct bands for the digested plasmids
- We hypothesized that CpG methylation was affecting the activity of EcoRI and decided to digest I716152, I716153, I716154, I716155, and K098010 for one hour with SpeI and I716390 for one hour with HindIII (I716390 is not consistent with standard Biobricks and does not have the XbaI, SpeI, or PstI cut sites)

July 13, 2009

- Two 0.8% agarose gels were run with the SpeI- and HindIII-digested plasmids as well as negative controls of undigested plasmids
- The correct bands were observed for I716390 (approximately 6.3kb) and K098010 (approximately 4.8kb), but not for I716152, I716153, I716154, or I716155
- We hypothesized that the enzyme was not being given enough time to act, so SpeI digestion was set up for those four Biobricks and left overnight in a heating block set at 37°C

July 14 2009

- Another 0.8% agarose gel was run with negative controls and once again, no correct bands were obtained
- The only avenue left unexplored is that our miniprep may not have been successful, so overnight cultures of I716152, I716153, I716154, and I716155 in 100 µg/mL ampicillin were set up

July 15, 2009

- I716152, I716153, I716154, and I716155 cultures were miniprepped and a PstI digestion was set up to leave overnight

July 16, 2009

- A 0.8% gel was run with the PstI-digested plasmids and negative controls and correct bands were observed for each of the four plasmids: I716152 (3.3kb), I716153 (3.0kb), I716154 (3.0kb), and I716155 (2.8kb)
- Since we have confirmed that we have isolated I716152, I716153, I716154, I716155, I716390, and K098010, we can begin our work in ligations

July 20, 2009

- An overnight culture of I716390 in 100 μg/mL ampicillin was started to monitor the absorbance over the week
- A sample of Top10 E. coli cells was obtained from Dr. Banfield's lab – we intend to perform a wavelength scan and compare it to the scan of the I716390 culture
- We received rehydrated DNA stocks of I13453, R0010, and I0500, glycerol stocks of J23119 and pSB1AT3 transformed into Top10, and several aliquots of chemically competent Top10 cells from Laboratory 1
- I13453 and pSB1A2 were heat-shocked with the chemically competent Top10 cells in order to grow them up and miniprep for ligations

July 21, 2009

- A wavelength scan was performed on the I716390 culture (no peak close to 412nm)*
- The optical density was measured and several serial dilutions were plated to take viable cell counts
- Single colonies were picked from the heat-shocked I13453 and pSB1A2 plates and restreaked
- Ligation schemes were made for our planned constructs: a Biobrick-format, constitutive heme (ABCD) plasmid, a constitutive HO-1 plasmid, and a constitutive HO-1 plus inducible heme (ABCD) plasmid
- An overnight culture of Top10 was set up (no antibiotics) to make electro-competent cells tomorrow
- Overnight cultures of I13453, pSB1A2, and J23119 in 100 μg/mL ampicillin and pSB1AT3 in 150 μg/mL tetracycline were made for a miniprep tomorrow

*After each wavelength scan the cultures had their media (LB) and respective antibiotics refreshed and again set for culture overnight

July 22, 2009

- I13453, pSB1A2, J23119, and pSB1AT3 were miniprepped
- Top10 electro-competent cells were made

- Wavelength scan was performed on the I716390 culture (peak at 406.6nm), optical density was measured, dilutions were plated, and cell counts from yesterday were recorded. Red colour (likely from heme precursors) production observed

July 23, 2009

- Minipreps of I13453, pSB1A2, J23119, and pSB1AT3 were cleaned up using the Qiagen PCR-Product Cleanup Kit
- A one-hour PstI digestion was performed for each of the above plasmids
- A wavelength scan plus all procedures from yesterday were repeated for the I716390 culture (peak at 408.6nm)
- An overnight culture of Top10 was set up (no antibiotics) for use in the wavelength scan tomorrow

July 24, 2009

- A 0.8% agarose gel showed the correct bands for our digested plasmids from yesterday: 2.3kb for I13453, 2.5kb for pSB1A2, 2.0kb for J23119, and 4.2kb for pSB1AT3 (both the backbone plasmids ran a bit large, but we are confident that we have obtained the correct plasmids)
- A wavelength scan plus all procedures from yesterday were repeated for the I716390 culture (peak at 407nm)
- As well, a wavelength scan was performed for the Top10 culture (no peaks in the heme range) and the wavelength scan for the I716390 culture was repeated with a blank of Top10 with no difference to the scan with a blank of LB
- We can conclude that the peaks we are getting with the I716390 culture are due to the plasmid and not the background strain

July 25, 2009

- A wavelength scan, optical density measurement, serial dilutions, and plate counts were performed for the I716390 culture (peak at 408.6nm)
- One-hour double digestions were set up as follows:
  - J23119, I716154, and I13453 were digested with EcoRI and SpeI
  - I716152, I716155, and K098010 were digested with XbaI and PstI
  - pSB4C5 was digested with EcoRI and PstI

- All digestions were cleaned up with the PCR Cleanup Kit and DNA concentration in each was measured using the spectrometer:
    - J23119: 1274 ng/mL
    - I13453: 6563 ng/mL
    - I716152: 9677 ng/mL
    - I716154: 10593 ng/mL
    - I716155: 15974 ng/mL
    - K098010: 9988 ng/mL
    - pSB4C5: 14255 ng/mL

July 26, 2009

- A wavelength scan, optical density measurement, serial dilutions, and plate counts were performed for the I716390 culture (peak at 406nm)
- Four ligations were prepared, with ratios of 2:2:1, 3:3:1, and 6:6:1 (Insert 1:Insert 2:Vector) for each:
    - Ligation 1: J23119 + I716152 + pSB4C5
    - Ligation 2: I716154 + I716155 + pSB4C5
    - Ligation 3: J23119 + K098010 + pSB4C5
    - Ligation 4: I13453 + I716152 + pSB4C5
- These ligations were set up with T4 ligase and volumes of each insert and vector calculated based on the determined DNA concentration in each sample
- Ligations were left at 16°C overnight

July 27, 2009

- A wavelength scan, optical density measurement, serial dilutions, and plate counts were performed for the I716390 culture (peak at 409nm)
- The three ratios for ligations 1 and 2 were electroporated into Top10 and plated on chloramphenicol plates

July 28, 2009

- A wavelength scan, optical density measurement, serial dilutions, and plate counts were performed for the I716390 culture (peak at 410nm)
- No colonies were observed on the chloramphenicol plates, meaning that our ligation did not succeed
- After discussion with Laboratory 1, we decided to try the ligations again with pSB1AT3 as the vector, since pSB4C5 did not work in their ligations either

July 29, 2009

- A wavelength scan, optical density measurement, serial dilutions, and plate counts were performed for the I716390 culture (peak at 412nm) -> TOOK 8 DAYS TO PRODUCE CORRECT PEAK

July 30, 2009

- A wavelength scan, optical density measurement, serial dilutions, and plate counts were performed for the I716390 culture (peak at 412nm)
- Based on this, we feel that we can conclude that heme is being produced by I716390, although it takes several days in Top10
- Overnight digestions were set up as follows:
  - J23119 and I716154 were digested with EcoRI and SpeI
  - I716152 and I716155 were digested with XbaI and PstI
  - pSB1AT3 was digested with EcoRI and PstI
- We digested 10μL of miniprepped plasmids instead of our usual 3μL in order to increase the chances of a successful ligation
- Since we are running low on rehydrated pSB4C5, we heat shocked the DNA into chemically competent DB3.1 cells obtained from Laboratory 1 and plated on chloramphenicol

July 31, 2009

- Instead of using the PCR Cleanup Kit on our digested plasmids, we decided to run a gel and perform gel extraction to purify the DNA using a QIAEX II Agarose Gel Extraction Kit

- A 0.8% agarose gel was loaded with digested plasmids J23119, pSB1AT3, I716152, I716154, and I716155, however, no band was observed in the well containing I716155
- Without I716155, we cannot do ligation 2, and so gel extraction was performed for bands at ~100bp for J23119, 3.5kb for pSB1AT3, and 2.3kb for I716152 only
- DNA concentration specs for the gel extractions were taken:
  - J23119: 3104 ng/mL
  - I716152: 20624 ng/mL
  - pSB1AT3: 15404 ng/mL
- Ligation 1 was set up and left at 16°C overnight
- Another gel was run with undigested I716155 plasmid to ensure that there is DNA present – a band was clearly visible and so it is unknown why the digested plasmid showed no band on the previous gel

August 4, 2009

- All three ratios from Ligation 1 were cleaned up with the PCR Cleanup Kit and electroporated into electro-competent Top10 cells, which were then plated on tetracycline

August 5, 2009

- No colonies were observed on the tetracycline plates
- After discussion with Laboratory 1, we concluded that the PCR Cleanup Kit might have washed away J23119, being such a small piece of DNA, which might be the reason that our ligations are not working
- In addition, we realized that each of our heme parts (A, B, C, D) have a stop codon at the end of the sequence, meaning that our construct would not produce heme without ribosomal binding sites
- A method for using PCR to extract the heme part from I716390 was explored with the idea of using primers with the proper enzyme cut sites on the ends, however this might be difficult since the piece we are trying to amplify is quite large – 4kb

- We decided to focus on the production of biliverdin using the heme construct (I716390) and the HO-1 construct (K098010) electroporated into a T7 polymerase-producing E. coli strain commonly used in our lab (NLM350)

- I716390 was electroporated into electro-competent NLM350 and plated on ampicillin

August 6, 2009

- Colonies grew on the ampicillin plates and were restreaked

August 11, 2009

- Triplicate overnight cultures of I716390 in NLM350 were made in 100 µg/mL ampicillin

- As well, an overnight culture of NLM350 was prepared (no antibiotics) for use as a negative control

August 12, 2009

- Wavelength scans, optical density measurements, serial dilutions, and plate counts were performed for the I716390 cultures (no peaks)

August 13, 2009

- Wavelength scans, optical density measurements, serial dilutions, and plate counts were performed for the I716390 cultures (peaks at 406nm, 405nm, and 407nm) and the NLM350 culture (no peaks in the heme range)

August 14, 2009

- Wavelength scans, optical density measurements, serial dilutions, and plate counts were performed for the I716390 cultures (peaks at 412nm, 412.6nm, and 412.4nm) and the NLM350 culture (no peaks in the heme range)

- Based on this, we conclude that heme is being produced in NLM350 at a much faster rate than in Top10 (3 DAYS VS. 8 DAYS)

August 17, 2009

- Overnight cultures of NLM350 (no antibiotics), NLM350 with I716390 in 100 µg/mL ampicillin and K098010 in 40 µg/mL kanamycin were prepared for use in protein gels and to make electro-competent cells

August 18, 2009

- Four flasks of LB inoculated with the NLM350, NLM350 + I716390, and K098010 overnight cultures were prepared as follows:
    - Flask 1: NLM350
    - Flask 2: NLM350 + I716390 to be induced with IPTG to overproduce heme
    - Flask 3: NLM350 + I716390
    - Flask 4: K098010
- Unfortunately, the cells did not grow to the point which was required for inducement with IPTG (OD=0.6), so these cultures had to be discarded
- Another flask of LB was prepared with NLM350 + I716390 as an inoculum to prepare electro-competent cells, but this flask also did not grow to the point at which electro-competent cells can be made (OD=0.8), so it also had to be discarded
- More overnight cultures were prepared to try these procedures again tomorrow

August 19, 2009

- Once again, the flask prepared with NLM350 + I716390 to make electro-competent cells did not reach an OD of 0.8 by the end of the day
- Flask 2 prepared for the protein gel reached an OD of 0.6 and was induced with IPTG, at which point all four flasks were left to shake in the water bath for an additional two hours to ensure that enough proteins were produced
- Cell lysates from each flask were prepared with Bugbusters and benzonase and loaded into a 12% acrylamide gel to run an SDS-PAGE protocol
- The gel was Coomassie stained overnight

August 20, 2009

- The completed gel showed no unique protein bands in either the NLM + I716390 (induced) or the NLM + I716390 wells, but we may have identified HO-1 at about 30kDa in the K098010 well (expected band at 33kDa)

August 21, 2009

- The NLM350 + I716390 electro-competent cells were made

August 24, 2009

- Both K098010 and I15008 were electroporated into the electro-competent NLM350 + I716390 and plated on ampicillin + kanamycin

- We realized that we may not have identified heme in the protein gel from August 21 because we used cell lysates rather than the culture supernatant (since heme is secreted)

- We must also confirm the presence of HO-1 by running another protein gel with a negative control

- Overnight cultures of NLM350, NLM350 +I716390 x 2, Top10, and K098010 were set up

August 25, 2009

- Colonies were observed on the ampicillin + kanamycin plates and restreaked

- Overnight cultures of NLM350 + I716390 + I15008 and NLM350 + I716390 + K098010 were prepared in 100 μg/mL ampicillin + 40 μg/mL kanamycin to begin the absorbance testing tomorrow

- Unfortunately, the NLM350 culture from last night did not grow, but we decided to run the protein gel as planned

- One of the NLM350 + I716390 cultures was induced with IPTG, and then all cultures were left to grow for an additional two hours

- Each culture was pelleted and the supernatant loaded into another acrylamide gel for SDS-PAGE

- Gel was Coomassie stained overnight

August 26, 2009

- No difference between the induced NLM350 + I716390 well and the uninduced NLM350 + I716390 well, indicating that heme is not present on the gel

- In addition, no difference was observed between the Top10 well and the K098010 well and there was no band at 30kDa, indicating that HO-1 is not present on the gel either

- Preliminary absorbance testing* Day 1, Check 1(11am):

  - NLM 350 with I716390 + K098010: 403.5 nm

- NLM 350 with I716390 + I15008: 403.0 nm
- NLM 350 with K098010: no peak

*the supernatant of pelleted sample as well as the supernatant of lysed samples were tested to determine if the products in question are secreted. Similar peaks were observed throughout the experiments suggesting protein secretion.

- Preliminary absorbance testing Day 2, Check 2 (2pm):
  - NLM 350 with I716390 + K098010: 403.0 nm
  - NLM 350 with I716390 + I15008: 404.0 nm
  - NLM 350 with K098010: no peak

August 27, 2009

- Preliminary absorbance testing Day 2:
  - NLM 350 with I716390 + K098010: 406.0 nm
  - NLM 350 with I716390 + I15008: 405.0 nm
  - NLM 350 with K098010: no peak

August 28, 2009

- Preliminary absorbance testing Day 3:
  - NLM 350 with I716390 + K098010: 413.4 nm
  - NLM 350 with I716390 + I15008: 410.0 nm
  - NLM 350 with K098010: no peak

August 29, 2009

- Preliminary absorbance testing Day 4:
  - NLM 350 with I716390 + K098010: 410.0 nm
  - NLM 350 with I716390 + I15008: 409.0 nm
  - NLM 350 with K098010: no peak

August 30, 2009

- Preliminary absorbance testing Day 5:

- NLM 350 with I716390 + K098010: 407.0 nm
- NLM 350 with I716390 + I15008: 406.0 nm
- NLM 350 with K098010: no peak

- Triplicate samples of the same three strains were set for overnight culture in order to collect large amounts of absorbance, concentration and viable cell count data and make the tests scientifically significant. The cultures were refreshed with LB media and their respective antibiotics after testing and set for culture overnight once again. Data is collected in an excel file over 5 days of experiment.

August 31, 2009

- Preliminary absorbance testing Day 6:
  - NLM 350 with I716390 + K098010: 408.0 nm
  - NLM 350 with I716390 + I15008: 408.5 nm
  - NLM 350 with K098010: no peak

September 1, 2009

- Preliminary absorbance testing Day 7:
  - NLM 350 with I716390 + K098010: 408.0 nm
  - NLM 350 with I716390 + I15008: 408.5 nm
  - NLM 350 with K098010: no peak

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