Notebook

University of Ottawa IGEM 2009

June 18, 2009

- Inoculated stab culture of pLEB590 from Dr. Takala's lab in 3 mL 10x sugar MRS broth. Incubating at 30°C overnight.
- Checked MRS plats of *L. lactis* strains; colonies have grown.

June 19, 2009

- Inoculates show no signs of growth
- Inoculated 5 samples of plated pLEB590 cultures in 5 mL of 10x sugar MRS broth.
- Inoculated 5 samples of pLEB590 from stab culture from Dr. Takala's lab in 5 mL 10x sugar MRS broth
- Growing up both trials, with two controls, over the weekend.

June 22, 2009

- Both trials of inoculates showed growth. Controls clean.
- Spun down cells; pellets appeared red. Resuspended in 1 mL, 750 uL or 500 uL miniprep solution (see www.openwetware.org for preparation), then carried out miniprep according to Sigma kit
- Digested miniprepped samples with EcoRI and EcoRV at 37°C for 1 hour
- Ran digested samples on a 1.25% gel. Expected bands at 460, 798 and 1838 bp.

June 23, 2009

- Gel showed the expected bands for all samples. Some streaking.
- Clearest bands appeared in the sample incubated in 1 mL of miniprep solution; protocol was altered to reflect this optimization

June 29, 2009

- Inoculated strains *A. xylinum* 53582 and *L. plantarum* NCIMB8826

July 2, 2009

- *L. plantarum* showed obvious signs of growth (media very murky). Glycerol stocked this inoculate.
- *A. xylinum* appears to have a translucent globule floating in the broth.

July 3, 2009

- Plated 7 uL of *L. plantarum* inoculate on MRS plates.
- Pelleted 1 mL of *A. xylinum* inoculate; removed supernatant. Repeated twice more for a total of 3 mL. Pellet was small, whitish and globular.
- Removed and plated 7 uL from the bottom of the tube and plated it.
- Both strains incubating at 30°C

July 6, 2009

- *L. plantarum* control plate contaminated. Re-plated from the glycerol stock
- *A. xylinum* plate showed potential signs of growth; allowing continued incubation overnight. Inoculate did not show any signs of growth; taking the same approach.

July 7, 2009

- PCR of pLEB590 for RFP expression (per tube):
  10 uL buffer
  1 ul dNTPs
  0.25 uL forward primer
  0.25 reverse primer
  4.25 uL DNA
  0.5 uL Phusion
  33.75 uL ddH_2O
- Ran PCR products on a 1.25% gel at 80V for 45 minutes. No bands appeared

July 8, 2009

- Re-performed pLEB590 PCR according to previous specifications
- Plated *A. xylinum* inoculate
- Inoculated *L. plantarum* in 3 mL MRS

July 9, 2009

- Inoculated 3 mL of *L. plantarum* in 30 mL MRS for preparation of competent cells
- Inoculates did not show enough growth; incubated overnight

July 13, 2009

- Attempting heat shock transformation of pLEB590 with competent *L. plantarum* cells. Ran six tubes:
  L1, L2: incubated in 1 mL lysozyme buffer for 3 minutes, spun down, then added DNA
  P1, P2: competent cells + DNA
  C1, C2: competent cells + ddH_2O
- After resuspension and incubation, samples L1 and L2 had a white precipitate on the bottom of the tube. Other samples were translucent with no precipitate.
- Incubating plates at 30°C overnight
- Inoculated 3 samples of *L. plantarum* in 10 mL MRS for use in a growth kinetics experiment.
- Plated RepA BioBrick strains on LB plates.

July 14, 2009

- Plates P1, P2, C1, C2 all showed signs of growth.
- Made plates with 120 IU/mL
- BioBrick strain with part pSB4A3 grew colonies; inoculated for minprep

July 15, 2009

- Kinetics experiment:

Time (h)	Sample 1 OD	Sample 2 OD	Sample 3 OD
0	0.0400	0.0213	0.0223
1	0.0451	0.0270	0.0346
2	0.0520	0.0345	0.0322
3	0.0588	0.0431	0.0437
4	0.0670	0.0657	0.0558
5	0.0822	0.0706	0.0654

- Placed samples in fridge overnight; to continue tomorrow

July 16, 2009

- Kinetics experiment continuation:

Time (h)	Sample 1 OD	Sample 2 OD	Sample 3 OD
5*	0.0914	0.0730	0.0722
6	0.1226	0.0949	0.0983
7	0.1582	0.1145	0.1219
8	0.1581	0.1141	0.1240
9	0.2119	0.1486	0.1601

- 5* indicates OD measurement taken upon removal from the fridge
- Heat-shock transformed 2 samples of *plantarum* with pLEB590

July 17, 2009

- Kinetics experiment:

Time (h)	Sample 1 OD	Sample 2 OD	Sample 3 OD
9*	0.1863	0.1325	0.1299
10	0.2130	0.1459	0.1544
11	0.2503	0.1696	0.1820
12	0.2904	0.1921	0.1882
13	0.3280	0.2197	0.2342
14	0.3509	0.2363	0.2532

- 9* indicates OD measurement taken upon removal from the fridge

July 22, 2009

- Confirmed amplification of the P45/NisI cassette
- *Xylinum* inoculates showed signs of growth after bench top incubation. Re-inoculated into 5 mL fresh media and plated a small amount
- Ended the kinetics experiment; 16 hours suggested for *plantarum* inoculate
- After PCR cleanup, DNA concentrations were very low based on measurements taken with the 4th floor spectrophotometer. This does not make sense and is consistent with improper readings from this spectrophotometer.
- Mutagenesis of pMC1 (per tube):
  32 uL ddH_2O
  10 uL buffer
  2.5 uL forward primer (10 pmol/uL)
  2.5 uL reverse primer (10 pml/uL)
  1 uL Phusion
  1 uL dNTPs
  1 uL DNA (~51 ng)

Program
  95°C 30s
  55°C 60s
  68°C 2.5 min
  Repeat 16 times

July 23, 2009

- Digested mutated pMC1 with SpeI or BamHI at 35°C for 30 minutes
- Ran digest product for 45 minutes on a 1% gel at 80V

- Undigested sample travelled the farthest→why? Expected bands did not show up
- Re-digested with SpeI and XbaI for one hour at 37°C
- Ran on a 1% gel at 80V for 45 minutes. Results not as expected; some bands undigested, others at the wrong spot
- Re-performed mutagenesis according to the same protocol
- Ran Victor's digest of P45/NisI with EcoRI on a 1% gel. Expected bands appeared

July 24, 2009

- Performed PCR clean up on mutagenesis products; pooled 3 samples into one tube to increase concentration
- Digested with 2 uL DpnI for 1 hour at 37°C; deactivated DpnI at 80°C for 20 minutes. Stored product at 4°C.

July 27, 2009

- Inoculates of *xylinum* are thick and jelly-like; two clear colonies have appeared on the plate.
- Split the initial inoculate into 5 mL inoculates, keeping the original
- Colony PCR of *xylinum* (per tube):
  10 uL buffer
  1 uL dNTPs
  2.5 uL forward primer
  2.5 uL reverse primer
  0.5 uL Phusion
  33.5 uL ddH_2O
  ~1 colony per tube

Program
  95°C for 5 min
  95°C for 1 min
  60°C for 20 s
  72°C for 5 s
  Repeat 35x
  72°C for 10 min

- Plated 7 uL of liquid from *xylinum* inoculate.
- Following colony PCR, digested product with EcoRI, then ran on a 1% gel at 80V for 45 min. No bands appeared.
- Used genomic extraction kit to extract *A. xylinum* genome, then performed PCR on the samples:
  10 uL buffer
  1 uL dNTPs
  2.5 uL forward primer
  2.5 uL reverse primer
  0.5 uL Phusion
  32.5 uL ddH_2O
  1 uL DNA

Program
98°C for 30s
98°C for 30s
60°C for 1 min
72°C for 5 min
Repeat above three steps 29x
72°C for 10 min

- Digested product with EcoRI at 37°C for 30 min, then ran on a 1% gel at 80V for 45 min.
- Gel showed extensive smearing, along with expected bands

July 28, 2009

- Inoculates of *A. xylinum* showed growth; plated sample grew a biofilm mat and many colonies
- Split and re-inoculated *A. xylinum* from second inoculate. Discarded initial inoculate.
- Attempted touch-down PCR on genomic extraction product. Set annealing temperature to T_m+10°C for first cycle, then decreased by 1°C for each successive cycle until T_m was reached, then cycle at that temperature for 19 cycles
  10 uL buffer
  1 uL dNTPs
  2.5 uL forward primer
  2.5 uL reverse primer
  4 uL DNA
  0.5 uL Phusion
  29.5 uL ddH_2O

July 29, 2009

- Digested PCR product with EcoRI at 37°C for 30 min
- Ran on a 1% gel at 80V for 45 min. No bands appeared; PCR unsuccessful
- *A. xylinum* plates have grown many very small colonies. All inoculates growing rapidly, as demonstrated by the high amounts of cellulose produced
- Performed PCR according to previous specifications, but with an annealing temperature of 56.3°C instead of 60°C.

July 30, 2009

- *E. coli* transformed with mutated pMC1 show growth on one plate after two days in the incubator; inoculated for confirmation miniprep
- Digested PCR product with HFEcoRI at 37°C for 30 min. Ran on a 1% gel for 45 min at 80V
- Control was contaminated. All other lanes have multiple bands where expected (and not) and smearing
- Digested PCR product with SacI for 1 hour at 37°C. Ran on a 1% gel at 80V for 45 min, then stored gel in the fridge.
- Performed two PCRs of the genomic extraction with an annealing temperature of 57°C and an increase in dNTPs. One using touch-down protocol, one using regular Phusion protocol
- Glycerol stocked the *A. xylinum* inoculate in 10% glycerol.

July 31, 2009

- Visualized SacI digest gel; bands appeared below 1kb.
- Digested touchdown PCR products with SacI at 37°C for one hour. None of the undigested samples showed any banding→touchdown PCR unsuccessful
- Attempted heat gradient PCR with the same specifications under Simon's PHU2STP program with a heat gradient.
- Transformation with pMC1: plates 1,2,3 and C1 are 0.05 ug/mL erythromycin. Plates 5,6 and C2 are 0.1 ug/mL erythromycin.

August 12, 2009

- Transformation unsuccessful; a mat of material appeared.

August 13, 2009

- Transformed strains showed some growth on 100 ug/mL erythromycin plates. Inoculated 4 colonies from 3 plates in 3 mL 100 ug/mL erythromycin LB

August 14, 2009

- 11/12 pMC1 inoculates showed signs of growth; control was clean
- Miniprepped the 11 positive inoculates. Those with low DNA concentrations were discarded.
- Digested all samples with SpeI at 37°C for 1 hour. Ran on a 1% gel at 80V for 45 min. Results inconclusive.

August 15, 2009

- Digested mutated pMC1 with SpeI and ran the product on a gel. Results still conclusive

August 17, 2009

- Performed a PCR according to previous specifications of the mutated pMC1 to increase concentration; correct band appeared on a 1% gel ran at 80V for 45 min.
- Concentrations measured on the 4th floor spectrophotometer are low, despite bright banding on the gel; machine obviously requires calibration.
- Digested Int/Attp from mutated pMC1 and P45/NisI with XhoI, then ligated at a 1:1 ratio overnight.

August 18, 2009

- Performed a PCR of the ligation product according to previous specifications with an annealing temperature of 65.6^{o}C and an elongation time of 1.5 min
- Ran product on a gel; successful band appeared.
- Concentration of PCR product, as measured on the 4th floor spectrophotometer, was low.

August 19, 2009

- Digested RFP part from the Registry with XbaI and XbaI + SpeI separately at 37°C for 1 hour.
- Ran on a gel; bands as expected.

August 21, 2009

- Prepared in-vitro DNA modification extraction

August 23, 2009

- Performed PCR of AB fusion part of cellulose synthase according to previous specifications.

August 24, 2009

- Extracted genomic DNA from *xylinum* again. Concentrations low and ratios poor→proteinase K might be bad

August 25, 2009

- Designed *plantarum*-optimized cellulose synthase construct.
- Received and plated RFP strain from HQ
- Plated *plantarum* competent cells to examine for contamination

August 26, 2009

- RFP strain grew colonies; inoculated into PMD for miniprep
- *Plantarum* plates from competent cells showed no growth; continuing to incubate
- Miniprepped RFP strains
- Replated *plantarum* from glycerol stock in an attempt to confirm or rule out contamination

August 28, 2009

- Inoculated two colonies of *plantarum* into 45 mL of MRS for use in the cell extract
- Inoculated 5 colonies from RFP+terminator ligation.

August 30, 2009

- Miniprepped inoculated colonies. Concentrations low, but pure.

August 31, 2009

- Attempted long-range PCR using DyNAzyme kit with 1.25 uL $MgCl_2$ per 50 uL reaction tube.
- Ran RFP + Term on a gel; expected band showed
- Prepared the *plantarum* extract according to the protocol. Sonicated each aliquot for 30s 3x each instead of protocol specifications
- Digested P45/NisI/RepA and GFP/Term constructs with XbaI and SpeI at 37^oC for 1 hour, followed by deactivation at 65^oC for 20 min.
- Ligated P45/NisI/RepA with GFP/Term in a 1:1 ratio overnight on the bench top.
- Ran DyNAzyme PCR product on 1% and 0.4% gels after head gradient PCR. Used λ-HindIII digest ladder as a reference.
- 3 samples showed possible positives.
- Attempted to optimize PCR with Mike by changing [$MgCl_2$]. Unfortunately, the same concentration was used for both reactions due to a miscommunication.

September 1, 2009

- Digested RFP/Term and GFP/Term with EcoRI.
- Ran cellulose synthase PCR products on a 0.4% gel. Two samples successful, along with some smearing.
- Confirmed P45/NisI/RepA/RFP/Term construct on a 1% gel. Multiple bands appeared on the GFP gel.
- Digested cellulose synthase and P45 cassette with XbaI and BamHI at 37^oC for 1 hour. Ligated at 1:1 and 1:3 (P45:CS) ratios
- Streaked pBLUESCRIPT from Corey on LB plates

September 2, 2009

- Ran PCR that Michael performed on a 0.4% gel; did not work as expected.

- Performed a PCR of the ligation product according to previous specifications, then ran on a 1% gel. Multiple bands appeared, where one was expected. P45 sample showed multiple bands.

September 8, 2009

- Modified the P45/RFP construct according to the in vitro protocol

September 11, 2009

- Checked concentrations of all samples after DNA modification. One sample had a different ratio than others, but all will be transformed.

September 17, 2009

- Diluted final RFP constructs with 10 uL TE to increase volume. Ran on a 1% gel. Band appeared, but was very faint.
- Attempted another PCR of genomic DNA with 1.75 uL $MgCl_2$ 50 ul reaction tube. Ran on a heat gradient of 50-55°C.

September 29, 2009

- Reconfirmed RFP construct, since previous gel was faint. Band appeared, but was still faint.

October 5, 2009

- Ran PCR of P45/Int ligation product on a 1% gel. 1 kb band appeared; expected a band at 4 kb. Ligation must be redone.

October 13, 2009

- Electrotransformed 50 uL of competent *plantarum* cells with 10 uL RFP construct. Dan plated and incubated.

October 19, 2009

- Control plate from electrotransformation was contaminated; must redo transformation.