Week 1 **04.06 - 10.06**

06.06

Plasmid Miniprep of phaCAB, pSB3T5, HlyBD, and phasin plasmids grown from overnight transformations. Contacted William Bothfeld at Northwestern University about his glucose toggle switch

Week 2 **11.06 - 17.06**

11.06

Prepared competent DH5α cells for InterLab study following iGEM protocols

Week 3 **18.06 - 24.06**

19.06

Transformed DH5α cells with BBa_R0040(Negative control), BBa_I20270(Positive control), BBa_J364000(Test device 1), BBa_J364001(Test device 2), BBa_J364002(Test device 3), BBa_J364007(Test device 4), BBa_J364008(Test device 5), and BBa_J364009(Test device 6) for Interlab.

20.06

Received plasmid maps from William Bothfeld to begin designing primers

Week 4 **25.06 - 01.07**
25.06

Created overnight cultures for InterLab

26.06

Followed InterLab protocols for 0h-6h timepoints and platereader measurements

Stab cultures arrived from William Bothfeld et al at Northwestern University and were grown in overnight cultures

27.06

Prepared minipreps of overnight cultures from Bothfeld et al.

29.06

Toggle switch was amplified with into two fragments at the site of the Illegal EcoRI site using two sets of primers (Fragment 1 Fwd, Fragment 1 Rev and Fragment 2 Fwd, Fragment 2 Rev) in Table 1 that added overlapping sequence between each other and pSB1C3 plasmid. Fragments of toggle switch were created with two separate PCR reactions following protocols for NEB Q5 polymerase.

Table 1. List of Primers designed to add overhangs for Gibson Assembly. Primers are for TetR:mCherry segment and LacI:crpp segment (Fragments 1&2 respectively),linear pSB1C3, and removing promoters from Biobricks for *pha* operon and *gfp* (BBa_K1149051 and BBa_I20270).

Primer name	Sequence	PCR Annealing Temp. (°C)
Plasmid Fwd	TACTAGTAGCGGCCGCTG	67.7
Plasmid Rev	CTCTAGAAGCGGCCGCG	67.7
Fragment 1 Fwd	cacatcgcaggaatcTTCAGGTACCAGGATCCG	60
Fragment 1 Rev	cgcggccgcttctagagAGATATCTTACTTATACAGCTCGTC	60
Fragment 2 Fwd	cctggtacctgaagaTTCCTGCGATGTGATATTGCTC	65.8
Fragment 2 Fwd	AGCGGCCGCTACTAGTAaactggcatgcggtcagtgcgt	65.8
PHA Fwd	ataTCTAGAcggcagagagacaatcaaatcatggcta	58
Biobrick Rev	actgcagcggccgctactagta	58
GFP Fwd	ataTCTAGAagtcacacaggaaagtactagatgcgtaaagg	58
VF2	tgccacctgacgtctaagaa	55
VR	attaccgcctttgagtgagc	55

Table 2. Thermocycling Conditions for cloning PCR using Q5 High-Fidelity DNA Polymerase. Annealing temperatures are listed in Table 1 for each set of primers.

Initial Denaturation	98 °C	30 seconds
33 Cycles	98 °C	10 seconds
	*see Table 1 for Annealing Temp.	15 seconds
	72 °C	30 seconds per kb
Final Extension	72 °C	2 minutes
Hold	16 °C	

PCR reactions using NEB Q5 High-Fidelity DNA Polymerase and the primers in Table 1 (PHA Fwd, Biobrick Rev, and GFP Fwd) were used to remove the promoters from the *phaCAB* operon and *gfp* parts (BBa_K1149051 and BBa_I20270).

Week 5 **02.07 - 09.08**

02.07

PCR products were loaded into 1% agarose gels with 6x loading buffer (NEB) and run at 120V for 45 minutes. Appropriate gel bands were excised and purified with QIAquick Gel Extraction Kit (Qiagen)

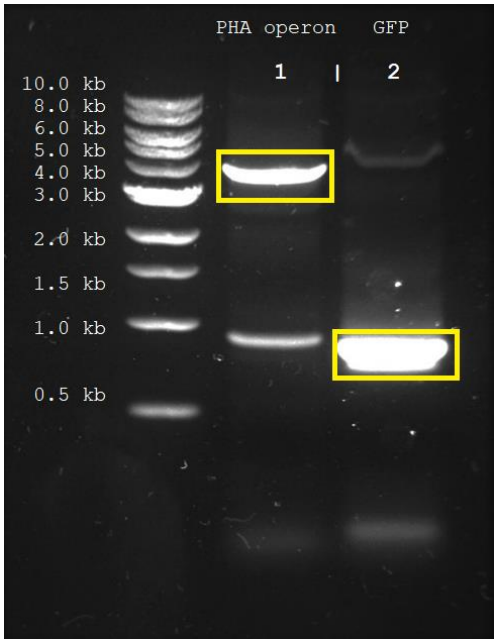


Figure 1. Agarose gel containing PCR products of the PHA operon and GFP without promoter. 1 kb DNA Ladder(NEB). Lane 1 contains PHA and GFP without promoter is in lane 2. Bands highlighted in yellow were selected, removed from gel, and purified.

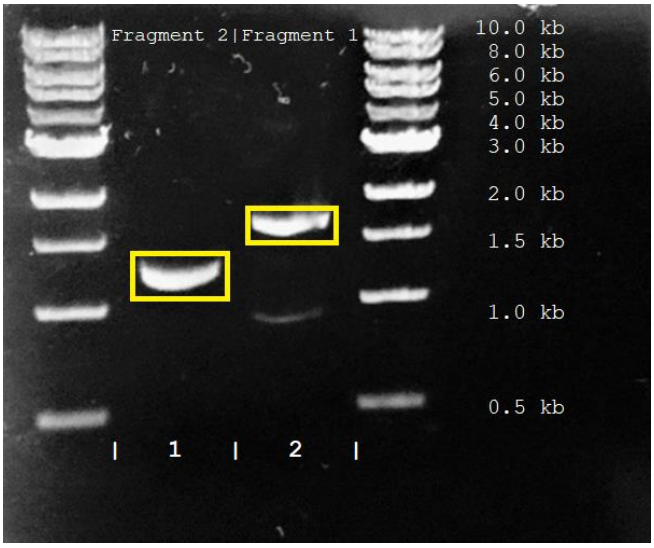


Figure 2. Agarose gel containing PCR products of Fragment 1 and Fragment 2 from pKDL071-TaraF with PHA ECAB operon. 1kb DNA Ladder(NEB). Fragment 1 PCR contains two PCR products. Bands highlighted in yellow were selected, removed from the gel, and purified.

A PCR reaction using NEB Q5 High-Fidelity DNA Polymerase and the primers in Table 1 (Plasmid Fwd and Plasmid Rev) were used to linearize pSB1C3. PCR was visualized with 1% agarose gel and cleaned with Qiagen PCR clean-up kit

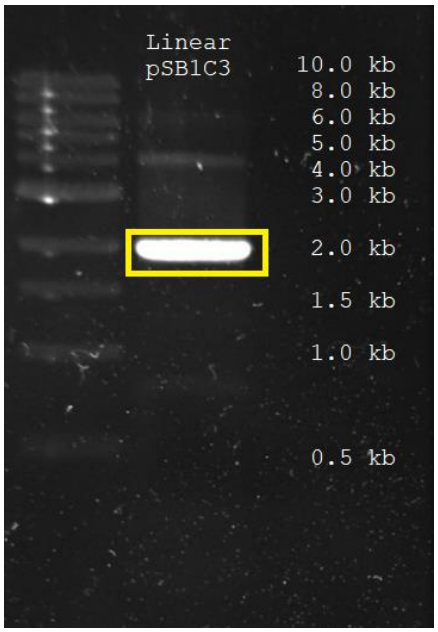


Figure 3. Agarose gel containing PCR product of linearized pSB1C3. 1 kb DNA Ladder(NEB). The band highlighted in yellow was selected, removed from gel, and purified.

03.07

Gibson assembly of the fragments and pSB1C3 backbone was set up using Gibson Assembly® Cloning Kit (NEB Catalog# E5510S). NEBioCalculator (NEB) was used to calculate the

molar ratio for Gibson assembly reactions. A total of 0.15 pmols of DNA fragments were added to re-action for a 1:1 ratio of Fragment 1 and Fragment 2 to linear pSB1C3. Initial re-actions were incubated at 50 ^{o}C for 15 minutes or 60 minutes as indicated in Table 3.

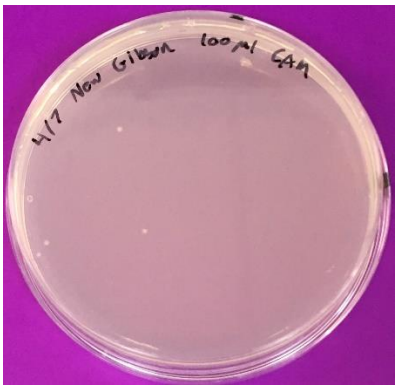
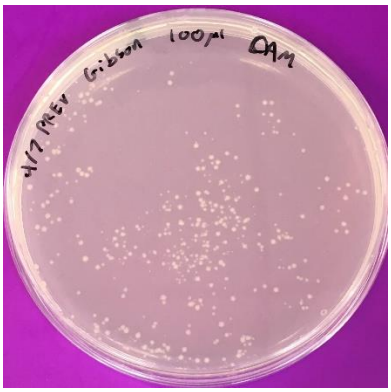
04.07

Second Gibson reaction was set up after seeing no growth on the plates. Later discovered the antibiotic was mislabeled for the plates. Both new and previous Gibson reactions were used for new transformation on chloramphenicol plates.

05.07

Many colonies formed on the plates from the Gibson Assembly reactions. Negative controls (backbone only) had more colonies than the normal reactions.

Table 3. Results of plating Gibson Cloning transformations on LB + chloramphenicol plates. A) Transformation of NEB Gibson Cloning kit cells using second Gibson reaction attempt incubated at 15 minutes. B) Transformation of NEB Gibson Cloning kit cells using second Gibson reaction attempt negative control incubated at 60 minutes. C) Transformation of DH5-alpha using first Gibson reaction attempt incubated at 15 minutes. Negative Control had showed a large number of colonies.

(A)	(B)	(C)
		

15 colonies were selected from the plates for colony PCR: Two red colonies from the negative control, two red colonies from each of the two Gibson Assembly reactions, and the rest were white colonies from the plates. Colonies were screened with colony PCR using the primers used to create both of the fragments

Table 4. Thermocycling Conditions for cloning PCR using GoTaq® Green Master Mix.

Initial Denaturation	95 ^{o}C	2 minutes
30 Cycles	95 ^{o}C	30 seconds
	55 ^{o}C	30 seconds
	72 ^{o}C	3 minutes 10 seconds
Final Extension	72 ^{o}C	2 minutes
Hold	16 ^{o}C	

06.07

Preformed 1% agarose gel analysis of colony PCR using Fragment 2 primers. Set up PCR using Fragment 1 primers of colonies that had products from Fragment 2

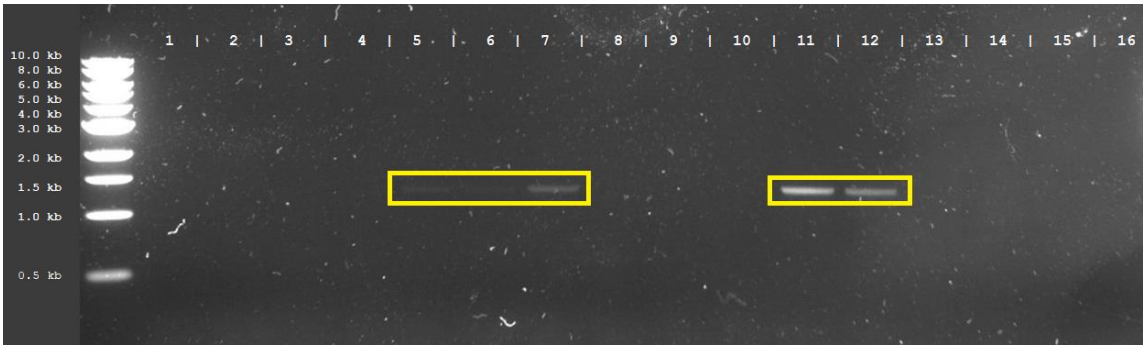


Figure 4. Colony PCR products of colonies taken from plates shown in Table 1. 1 kb DNA Ladder(NEB). Lanes 1 and 2 contained red colonies taken from negative control while lanes 3 and 4 contained red colonies from the Gibson reactions. Lanes 5-16 contain white colonies from the plates. Lane 16 was water as template. The primers used in PCR were the same to clone out Fragment 2. Lanes 5-7, 11, and 12 all contain bands at ~1.3 kb.

07.07

Preformed 1% agarose gel analysis of colony PCR using Fragment 1 primers

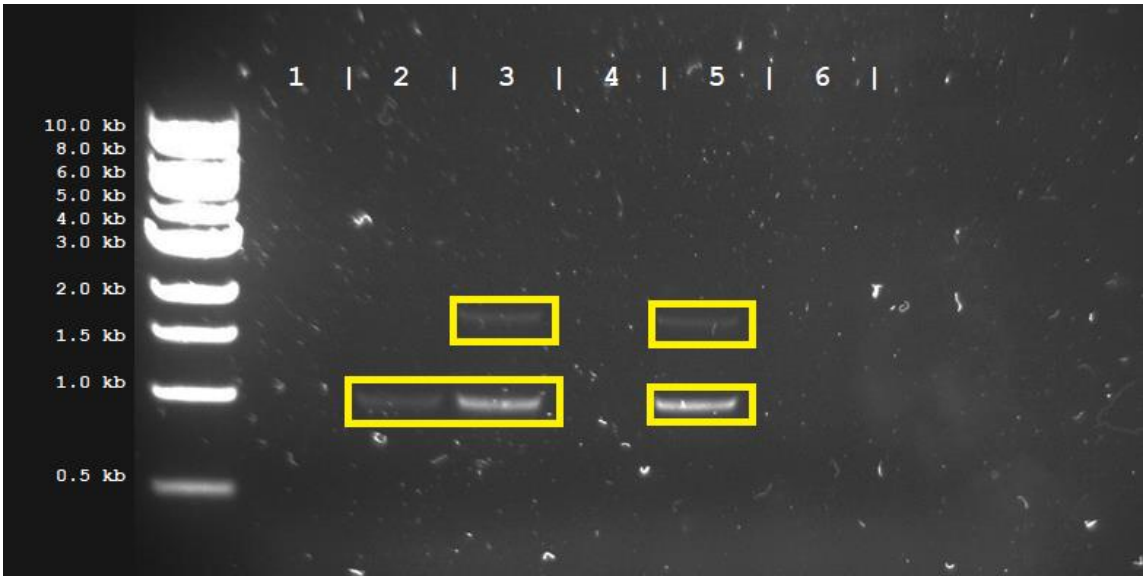


Figure 5. Colony PCR of plated colonies from **Figure 4** using primers used to clone Fragment 1. 1 kb DNA Ladder(NEB). Lanes 1-5 contain the colonies from **Figure 4** and Lane 6 contains (-) PCR control. Three colonies showed bands with two of them showing two bands at the same locations as in **Figure 4**.

Week 6 **09.07 - 15.07**

09.07

Overnights of the three positive colonies were grown

10.07

Minipreps of the 3 colonies were digested with EcoRI and PstI and analyzed on 1% agarose gel to see insert length

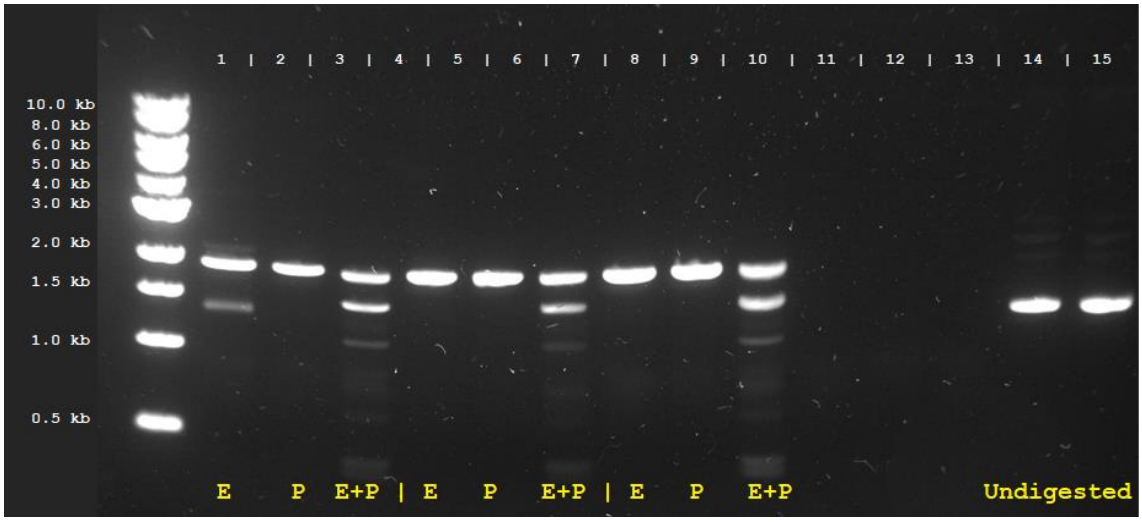


Figure 6. Restriction enzyme digest of minipreps from the overnight cultures in Figure 5. 1 kb DNA Ladder(NEB). Each of the three colony's plasmid minipreps (300 ng of DNA) were digested with EcoRI, PstI, and a double digest of the two enzymes denoted with E, P, and E+P respectively. Undigested plasmid was loaded into lanes 14 and 15. All of the digests show bands at ~2000 bp but digests containing EcoRI show additional smaller bands fragments. This suggests that the EcoRI reactions had some unspecific binding patterns and over digested the plasmid products.

Overnight colonies for Interlab study for repeat of experiment

11.07

Because of the unexpected banding patterns in the first reaction, it was decided to re-digest the plasmids for only one hour

Figure 7. Restriction enzyme digest of minipreps from the overnight cultures in Figure 5. 1kb DNA ladder from Promega. Each of the three colonies (300 ng of DNA) were digested for overnight with EcoRI, PstI, and a double digest of the two denoted with E, P, and E+P respectively. Lanes 1-3 contain colony N1, 4-6 contain N2, 7-9 contain P3. Lanes 12-14 contain undigested plasmids from N1, N2, and P3 respectively. Single digest using EcoRI was loaded into lanes 1,4, and 7. PstI digests were loaded into lanes 2, 5, and 8. Double digests were loaded into lands 3, 6, and 9.

Measurement of Interlab controls and test devices and serial dilution of controls. Serial dilutions were also plated and grown overnight at 37 ^{o}C

12.07

CFU from inter lap was counted. Colony PCR of colony plasmid minipreps was done to confirm that there was no insert in each plasmid

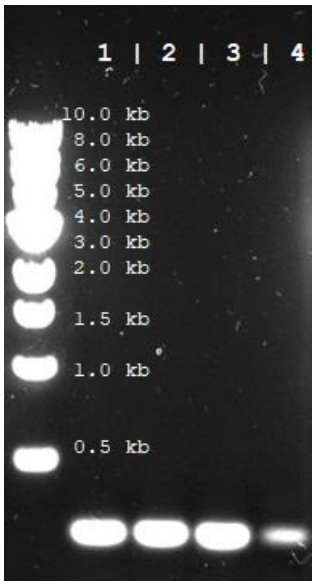


Figure 8. Colony PCR of colonies using VF2 and VR primers**.** 1kb DNA ladder from Promega. Lanes 1, 2, and 3 contain colony PCR of three colonies showing positive colony PCR and (-) PCR control using the VF2 and VR primers.

Week 7 **16.07 – 22.07**

18.07

More colonies were selected from plates containing the Gibson reactions. Colony PCR was performed using GoTaq® Green Master Mix

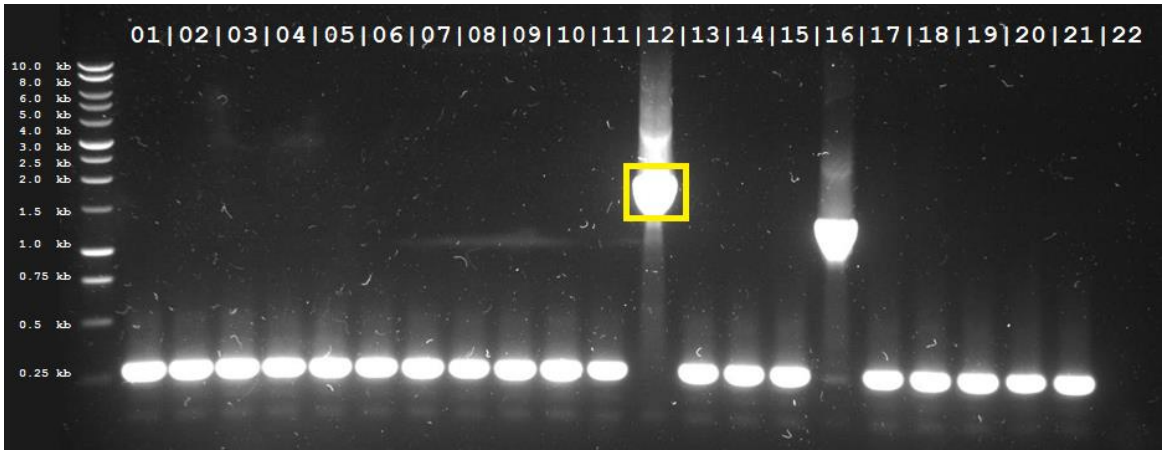


Figure 9. Colony PCR of more colonies from the Gibson reactions plated in Table 1 using the VF and VR2 primers. 1kb DNA ladder from Promega. Lane 16 contains the (+) control(pSB1C3:rfp) and lane 22 contains (-) PCR (dH2O) control. Lane 12 contains a band of ~ 1.7 kb while lane 16 contains a band of ~ 1 kb.

19.07

Overnight culture of colony with insert in pSB1C3

20.07

Restriction enzyme digest of minipreps from the overnight culture

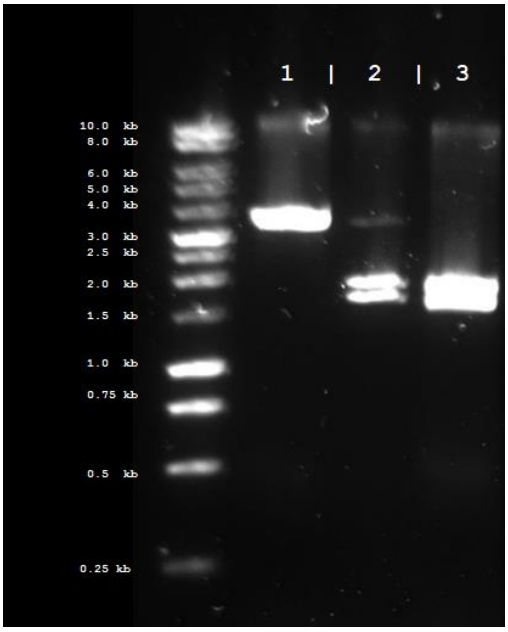


Figure 10. Restriction enzyme digest of minipreps from the overnight culture lane 12 of Figure 9. 1kb DNA ladder from Promega. The miniprep was digested with EcoRI, and double digests using EcoRI + PstI, and SpeI + XbaI in lanes 1, 2, and 3 respectively. Lanes 2 and 3 contain two bands each at ~2 kb and ~1.7kb while Lane 1 contains one band at ~ 4.7 kb.

Week 8 23.07 - 29.07

23.07

Linearized plasmid and insert using the plasmid Fwd primer and Fragment 1 Rev primer

24.07

Because half of the toggle switch was inserted into the plasmid, another Gibson reaction using a linear plasmid and Fragment 1 could be used as the vector and Fragment 2 could be added in a higher ratio

25.07

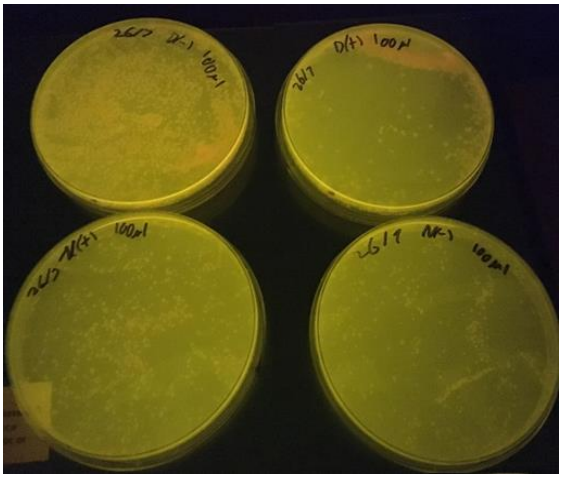


Figure 11. Plated colonies of Gibson reaction using linearized pSB1C3:Fragment 1 and Fragment 2. Negative controls of transformations using only linear SB1C3:Fragment 1 contain similar number of colonies compared to reaction using SB1C3:Fragment1 + Fragment 2. Plates are imaged on a light box with emission centered around 470 nm.

Week 9 30.07 - 05.08

30.07

Picked 10 more colonies for colony PCR but unable to get second insert in plasmid

Week 10 06.08 - 12.08

Dissertation write up

Week 11 13.08 – 19.08

Dissertation write up

Week 12 20.08 – 26.08

22.08

Digestion of all parts (phaR, phaR-phasin, phaR-phasin-HlyA) with pSB3T5 backbone and of pSB1C3 vector with EcoRI and PstI

Table 5. Protocol followed for double digestions of parts in pSB3T5 and of pSB1C3 plasmid using Promega products.

dH_2O	15.8 uL
Buffer H	2 uL
BSA	0.2 uL
DNA	1 uL of 1 ug/uL
EcoRI	0.5 uL
PstI	0.5 uL

23.08

Ligation of fragments into EcoRI and PstI digested pSB1C3 backbone and transformation

24.08

Cells did not grow so Ligation was done again

Week 13 27.08 – 02.09

28.08

(+) transformations control showed our second batch of competent cells were not working

29.08

Ligation was repeated with NEB Top 10 competent cells

Week 14 03.09 – 09.09

03.09

Colonies were selected from each place and grown in overnight cultures

04.09

Minipreps were prepared from overnight cultures. Each insert was analyzed with EcoRI and PstI digestion as shown in Table 5

05.09
Gel result showed inserts that are correct sizes for phaR and phaR-phasin-HlyA

06.09

Overnight cultures for phaR-Phasin colonies

07.09

Digestions of phaR-Phasin minipreps

Week 15 10.09 – 16.09

11.09

Overnight culture of Part:BBa_K390501 phasin-HlyA part from distribution kit for sequencing

12.09

Miniprep of phasin-HlyA part. Prepared 5x M9 media, 1M $MgSO_4$, and 1M $CaCl_2$ for 5 L bioreactor and gas chromatography analysis of plastic. Shipped phaR, phaR-phasin, phaR-phasin-HlyA, BBa_K390501 phasin-HlyA, phasin-HlyA without stop codon, and Bktb for sequencing at Dundee. Prepared three Bktb colonies for overnight.

Table 6. 5x M9 media recipe.

$Na_2HPO_4 \cdot 7H_2O$	64g
KH_2PO_4	15g
NaCl	2.5g
NH_4Cl	5g
Adjust to 1000 mL and Autoclave separate from 1M $MgSO_4$ and 1M $CaCl_2$	

13.09

The three Bktb overnight cultures were placed in fresh LB for 5 hours before being placed into M9 media with 3% glucose, 2 mM $MgSO_4$, 10 uM Acetic acid, and 0.1 mM $CaCl_2$. These cells were placed at 37 ^oC to grow overnight

14.09

Bktb overnight cultures in M9 media had propionic acid added once OD_{600} reached 0.8 for a final concentration of 8 mM propionic acid. These cultures were placed 37 ^oC to grow over the weekend

Week 16 17.09 – 23.09

17.09

Cells were spun down, supernatant was removed, and pellets were lyophilized for GC analysis

19.09

Dried cells and PHBV standards were prepared for GC analysis along with negative controls. Samples included PHBV standard, extracted plastic, lyophilized cells, and negative control. More M9 media was made for 5 L bioreactor run. Media containing Pot ale was also made using 5x M9 salts

Table 7. Recipe for growing cells in Pot Ale to produce plastic.

M9 salts	1x
glucose	3%
$MgSO_4$	2 mM
$CaCl_2$	10 uM
Acetic acid	and 0.1 mM
Filtered Pot ale	Fill to needed volume

21.09

5 L Bioreactor was set up using M9 salts and 0.1 mM acetic acid. GC results were inconclusive for PHBV. PHV did not show in the standard

22.09

Bioreactor growth seemed slow. Propionic acid was not added as the OD_{600} was stuck at 0.04

Week 17 24.09 – 30.09

24.09

Bioreactor was halted as cells did not grow over the weekend

Figure 12. 5 L Bioreactor containing M9 media with 3% glucose holding dead Bktb cells. Bktb cells did not grow past an OD_{600} of 0.04.

28.09

More cells were grown overnight in M9 media for GC analysis

29.09

Propionic acid was added to overnight cultures when OD_{600} reached 0.8

Week 18 01.10 – 07.10

01.10

150 mL overnight Bktb culture was grown for bioreactor set up in LB. 5x LB and glucose were prepared for new bioreactor run in LB media

02.10

Overnight was placed into 350 mL of LB for 5 hours before being spun down to 50 ml to inoculate bioreactor

03.10

Six cultures of Bktb in M9 media and 1% glucose were set up with three containing Pot ale and the other three containing water. Colonies were set up to shake at 37 ^{o}C overnight

04.10

OD_{600} readings of both pot ale experiment and bioreactor were taken throughout the day

05.10

Pot ale experiment halted at the end of the day and cells placed into 4 ^{o}C for extraction

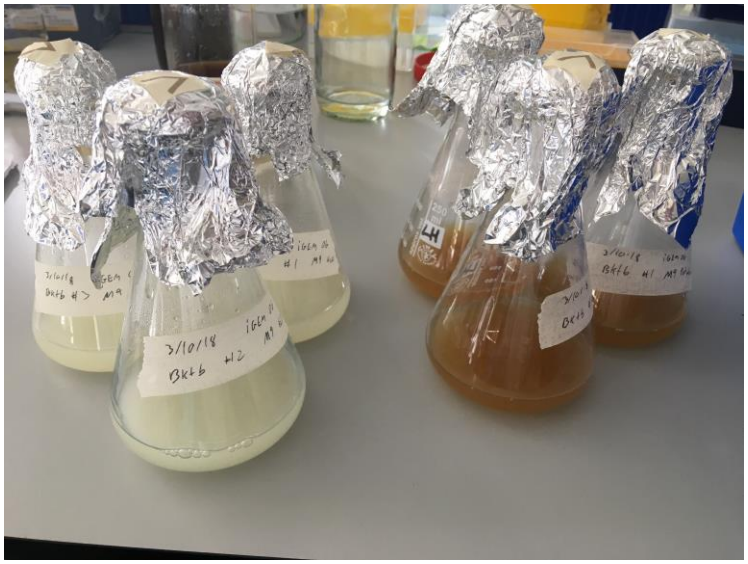


Figure 13. Three Bktb cell cultures growing in M9 with pot ale (Right) and without pot ale (Left). Three separate Bktb cultures were used to inoculate two cultures each. Cultures were grown at 37 ^{o}C for three days.

Table. OD_{600} of Bioreactor run containing Bktb cells grown in LB media and 3% glucose. 8 mM propionic acid was placed in at 19:42 hours of the run.

Table 7. OD_{600} of bioreactor grown over 72 hours. 8mM propionic acid was added at 19 hours 42 minutes.

Time	OD600
0:00	0
18:00	0.7
18:52	0.73
19:42	0.76
24:00:00	0.99
42:00:00	1.55
44:00:00	1.59
45:30:00	1.62
47:50:00	1.61
66:30:00	1.67
68:15:00	1.69
72:00:00	1.73

Week 19 08.10 – 14.10

08.10

Bioreactor was shut off

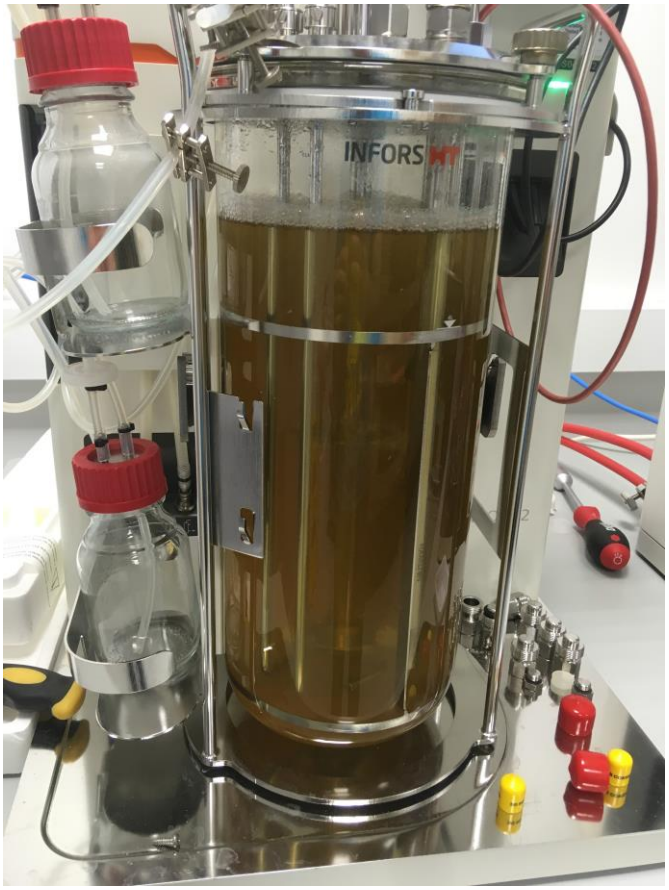


Figure 13. 5 L bioreactor growing Bktb cells in LB with 3% glucose.

12.10

Extraction of plastic began

Week 20 15.10 – 17.10

15.10

Finished extraction of plastic

16.10

Plastic taken to GC analysis as well as melting point analysis