LAB DIARY FOR SUPERCOILIOLOGY PROJECT

1st JULY 2011 until 16th JULY 2011

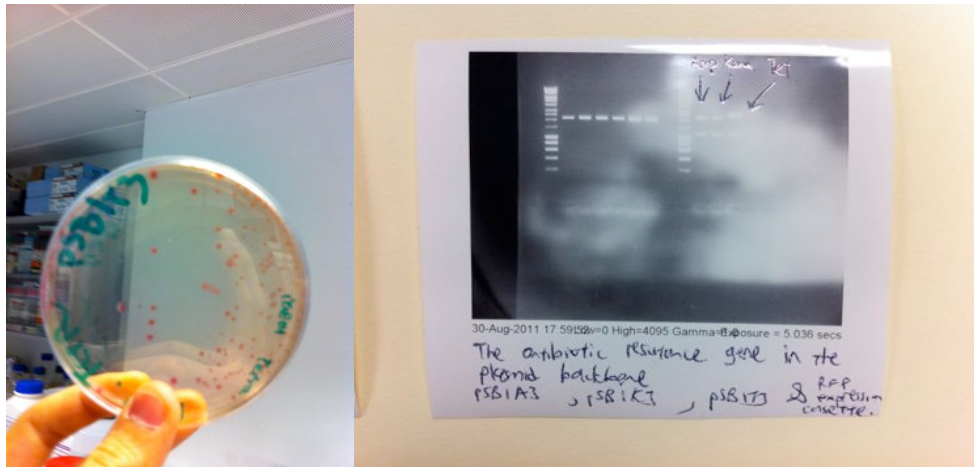
Initially we have two weeks of training session in which our instructors showed us the protocols for making the competent E.coli cells , digestion of DNA and the protocols for agarose gel electrophoresis.

We familiarize ourselves with the lab equipments and move on to start our iGEM experiments!

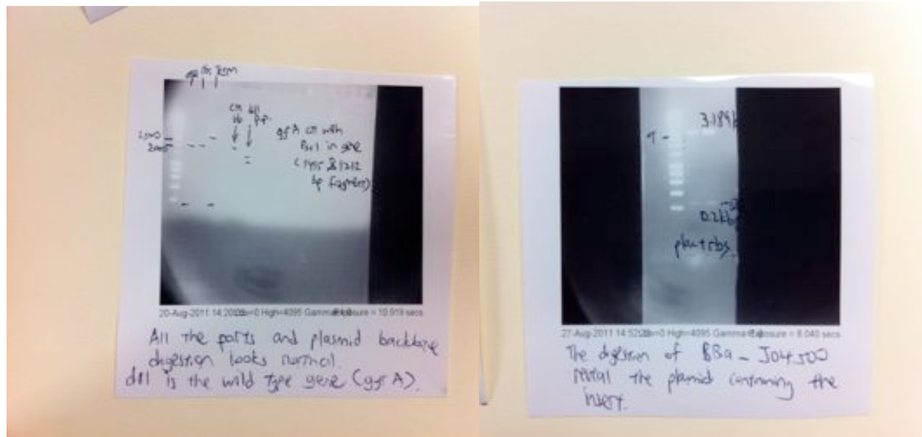
18th July 2011 until 10th AUGUST 2011

First , we had made competent TOP10 cells which was supplied by Oriana (refer to the Protocol section). We have also identify the type of promoters and parts which we intended to use by using the parts registry . Then we transformed the competent cells with the following plasmids from the kit plate:

1)BBa_K206000 (strong pBAD promoter) in 2011 iGEM kit plate 4 (Well 8G)
2)BBa_B0034 (RBS) in 2011 iGEM kit plate 1 (Well 2M)
3)BBa_B0015 (double terminator) in 2011 iGEM kit plate 1(Well 23L)
4)BBa_J04500 (IPTG inducible promoter with RBS) in 2011 iGEM kit plate 4(Well 12A)



After transformation and overnight cultures , we miniprep the plasmids and check whether did they contain the correct inserts and plasmid backbones. We later run the samples on the gel together with the digested linearized pSB1C3 and gyrase genes from the planned PCR.



By using EcoCyc and NEB cutter V2.0 , we have found our that there was a Pst1 restriction site in GyrA gene at 1212th bp position while and EcoR1 restriction site was also present at 6th bp position in GyrB gene.

Using Eurofins operon (Prof Ward had kindly allowed us to use his account), we designed the appropriate primers which include the standard EcoR1 and XbaI restriction sites upstream of the ORFs while another SpeI and PstI restriction sites in the downstream of the ORFs. Here are the primers we used:

gyrA (For) TAG TTC GAA TTC TCT AGA ATG AGC GAC CTT GCG AGA GAA AT (41 bp)
gyrA (Rev) ATC ATC CTG CAG ACT AGT TTA TTC TTC TTC TGG CTC GTC GTC (42 bp)

gyrB (For) TAG TTC GAA TTC TCT AGA ATG TCG AAT TCT TAT GAC TCC TCC (42 bp)
gyrB (Rev) ATC ATC CTG CAG ACT AGT CTC GCA TGG TTA GCG CCA TTA (39 bp)

PCR mixture (50 µl) of gyrA and GyrB from TOP10 E.coli genome

Volume (µl)	Solutions
32.5	dd H_2O
10.0	5X HF buffer
1.0	dNTP mixture
2.5	Forward primers (10 µM)
2.5	Reverse primers(10 µM)
1.0	Template DNA *
0.5	Phusion DNA polymerase

*The template DNA was made by suspended a small colony of TOP10 E.coli into 100 µl of ddH_2O and followed by vortex for 15 seconds.

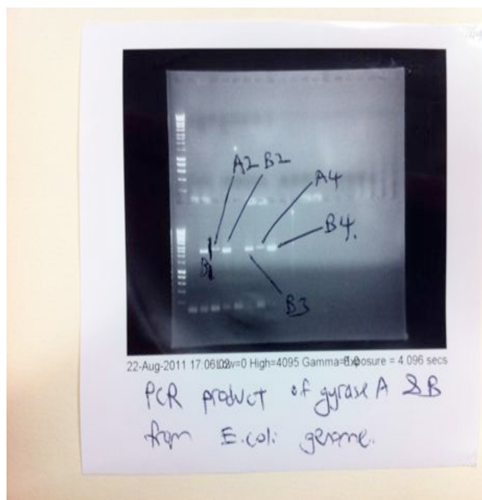
We centrifuge for all of the materials to be collected at the bottom of the PCR reaction tubes and incubate our PCR mixture in a PCR machine(or thermomixer).

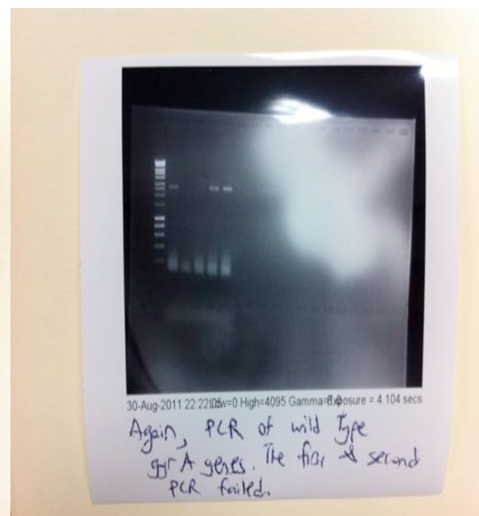
Cycling conditions:

Stage 1	1 Cycle	98 °C	30sec
Stage 2	30 Cycles	98 °C	10sec
		65 °C	30sec
		72 °C	1min 30sec
Stage 3	1 Cycle	72 °C	10min
Stage 4*	----	4 °C	------

*Stage 4 is just to put the reaction to a hold.

We have several failed attempts with this as our primer and template concentrations were too high. We then made some corrections such as serial dilutions of the template DNA and perform the PCR reaction again. As such in the end we have succeed in extracting and amplifying our GyrA and GyrB genes.





10th AUGUST 2011 until 24th AUGUST 2011

Next , we ligated the GyrA and GyrB PCR products into pSB1C3 backbones.

Digestion of pSB1C3 plasmid backbone(BBa_J04450) , GyrB and GyrA PCR products:

Materials:	Volume(µl)
DNA (about 500ng)	7.5
The first restriction enzyme	1.0
The second restriction enzyme	1.0
10X NEB Buffer 2	5.0
100X BSA	0.5
ddH_2O	35

The digestion mixture will the be incubated in a 37 °C water bath for 15 minutes and followed by heat inactivation at 80 °C for 20 minutes.

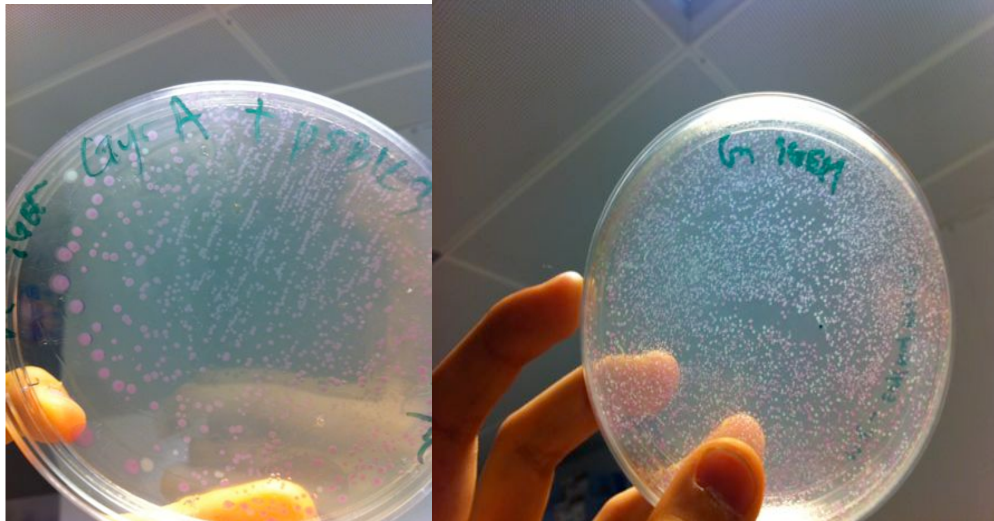
For the first digestion , we used GyrA and digested the PCR product with E coR1-HF(E) and SpeI(S) restriction enzyme. In this way the wild type GyrA gene will not be cleaved by the restriction enzymes .
For the second digestion , we used the miniprep BBa_J04450 plasmids and digested the plasmid with E and S. This digested backbone will be used to ligate GyrA gene into the pSB1C3 plasmid backbone.
For the third digestion , we used GyrB PCR product and digested it with XbaI(X) and PstI (P) . In this way , the wild type GyrB will not be cleaved by the restriction enzymes.
For the fourth digestion, we used the miniprep BBa_J04450 plasmids and digested the plasmids with X and P. This digested plasmid backbone will be used to ligate GyrB instead into the pSB1C3 backbone.

Ligations of GyrA and GyrB into pSB1C3:

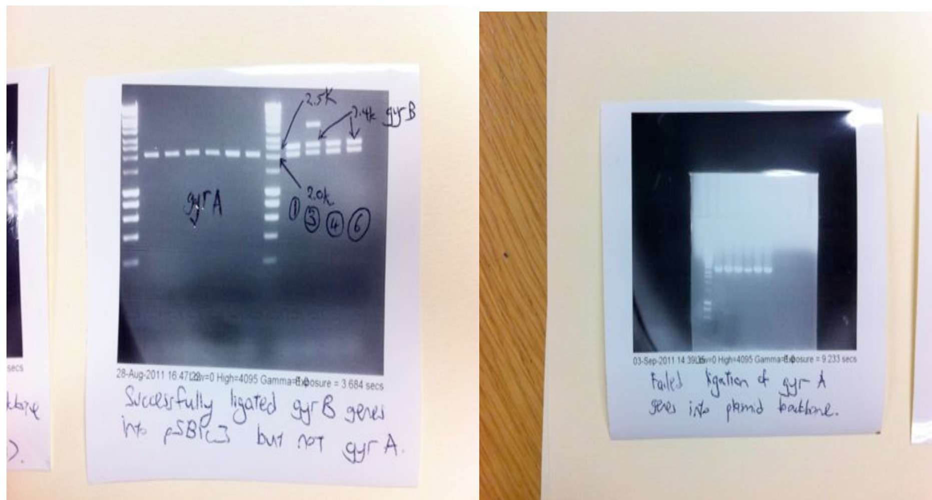
Materials:	Volume (µl)
Digested PCR products	5.0
Plasmid backbone(pSB1C3)	2.0
10X T4 DNA ligase buffer	2.0
T4 ligase	1.0
ddH_2O	10.0

The ligation mixtures will then be incubated at room temperature for 10 minutes and heat inactivated at 80 °C for 20 minutes.

After we carry out transformation of the ligated plasmid into our competent TOP10 E.coli cells to amplify the ligated plasmid products.



We also carried our digestion (GyrA with E and S while GyrB with X and P) of the miniprep from the overnight cultures to check whether did we have the correct insert.



From the several miniprep of the overnight cultures which we made , we have successfully ligate the GyrB gene into pSB1C3 while the ligations of GyrA failed. In our following attempts we had tried to ligate the GyrA PCR products into a TOPO blunt end plasmid backbone (the second photograph). We did 6 of them and unfortunately all of the TOPO plasmid backbones self-ligated.

Zero blunt TOPO PCR cloning method:

Materials	Volume (µl)
Fresh PCR product (GyrA)	4.0
Salt solution	1.0

ddH_2O	Added to a final volume of 5 µl
PCR Blunt TOPO plasmid backbone	1.0
Total volume	6.0

The reaction mixture was then incubated at 25°C for 20 minutes and followed by placing the tubes on ice. After that transformation was carried out to transformed the ligated plasmid into the provided competent cells in the kit.

<u>25th AUGUST 2011 until 7th SEPTEMBER 2011:</u>

Site directed mutagenesis(SDM):

Thinking that since we already have GyrB ligated into pSB1C3 we ran a site directed mutagenesis PCR reaction on the plasmid by using two different protocols (the Stratagene kit and the one with Phusion DNA polymerase) .

By using EcoCyc and eufins operon again , we had designed the appropriate primers which removed the 6th bp EcoR1 site in GyrB ORF.

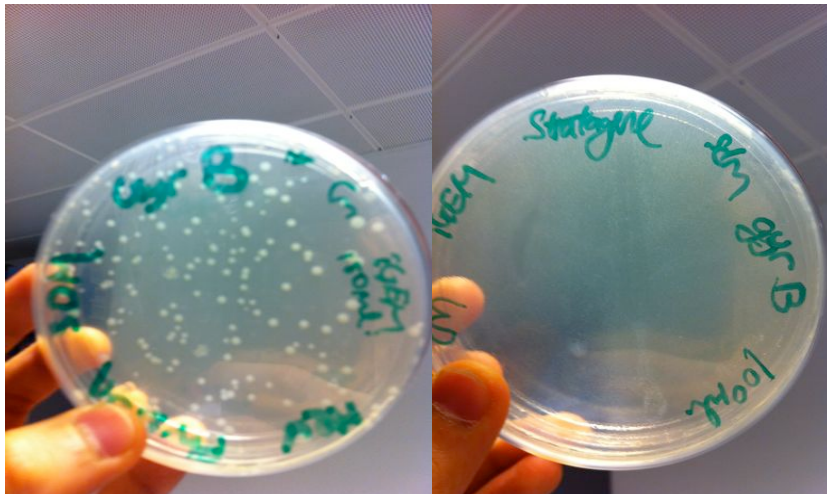
The SDM protocols involving phusion DNA polymerase was essentially the same as the one provided previously with the primers containing the mutated sequence , while the following protocols are for the Stratagene SDM method.

Stratagene site directed mutagenesis protocol:

Materials	Volume (µl)
10X reaction buffer	5.0
Template plasmid (6ng/ µl)	5.0
Forward primer (100ng/ µl)	1.0
Reverse primer (100ng/ µl)	1.0
dNTP mix	1.0
Quick Solution reagent	1.5
ddH_2O	34.5

After that 1 µl of Quick Change enzyme was added and follow by the following cycling reactions.

Stage 1	1 Cycle	95 °C	2min
Stage 2	18 Cycles	95 °C	20sec
		60 °C	10sec
		68 °C	2min 30sec
Stage 3	1 Cycle	68 °C	5min
Stage 4*	----	4 °C	------



The colonies from the Phusion SDM reaction yielded good number of colonies but we have a different morphological growth of E.coli from the Stratagene kit.

We picked 6 colonies from each plate and did overnight cultures for each of them. On the next day , we did miniprep and to our disappointment all of the colonies did not give us the desirable results .

Previously , we have tried to ligate the GyrA and GyrB genes into the linearized plasmid backbone provided. However after several failed attempts , we decided to use the BBa_J04450 RFP expression cassette instead to ligate the biobricks.

We had our meeting with our instructors and supervisors to get advices on which subproject should we focussed on since the wiki freeze deadline was closing in . They told us to focus on the GBS biobricks which we made and did all the necessary characterisation such as shake flask fermentation to get the E.coli growth profile and the GFP expression level from the stress light devices.

Please do refer to our GBS project section for our main work.

iGEM

UCL