Laboratory Records: Cloning of Synthetic Catabolic Pathways of Fluorene and Phenanthrene Under the Control of an Inducible Promoter

Table of Contents

1- PURPOSE 2
2- CLONING STRATEGY 2
3- GENE CHARACTERISTICS 2
4.1. FLUORENE 2
4.1. PHENANTHRENE 3
4- DNA PREPARATION 4
4.2. MATERIALS 4
4.3. DNA PREPARATION 4
5- VECTOR PREPARATION 5
4.1. MATERIALS 5
4.2. METHODS 5
4.2.1. Preparation of recipient vector (insert 1) 5
4.2.2. Preparation of insert 2 6
4.2.3. Gel purification of DNA fragments 7
4.2.4. Ligation 8
4.2.5. Transformation 9
4.2.6. Transformation Results 10
4.2.7. Clone Verification 11
6- GENERATION OF CONSTRUCTS BL-21 17
6.1. MATERIALS 17
6.2. METHODS 17
7- GLYCEROL STOCK 19

1- PURPOSE

Cloning of fluorene and phenanthrenecatabolic pathway for under the control of an inducible promoter and expression in *E.coli*.

2- CLONING STRATEGY

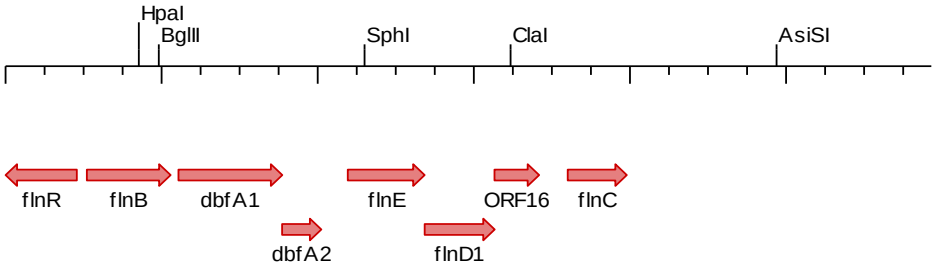
The catabolic pathway for fluorene and phenanthrene were synthetized as polycistronic operons with the codon optimized for expression in *E.coli*.
The catabolic pathways for fluorine and phenanthrene were split into 2 parts for several reasons:
(i) To facilitate the synthesis of the genes (cost-effective and in a timely manner);
(ii) To ensure a good level of expression of the polycistronic genes;
(iii) To determine if there are orientations of the polycistronic operons that may be more favorable for expression; and
(iv) To minimize toxicity issues that may arise when the full pathway is synthetized with all the genes.

Both parts are cloned into pUC57. One plasmid designated insert 1 contains only one HindIII site. The second part of the catabolic pathway, designated insert 2, carried 2 HindIII sites flanking the insert. Insert 2 is digested with HindIII and this fragment is ligated into the vector carrying insert 1 after digestion with HindIII and dephosphorylation. The product of the ligation is transformed into E.coli DH5a. The clones are analyzed to determine if they have an insert and its orientation. The correct clone is then transformed into E.coli BL21 to allow for high level of expression. During growth, IPTG is added to induce the expression of the polycistronic genes. The culture may be grown in minimal medium with fluorine or phenanthrene as a sole source of carbon to determine if there the catabolic genes are functional.

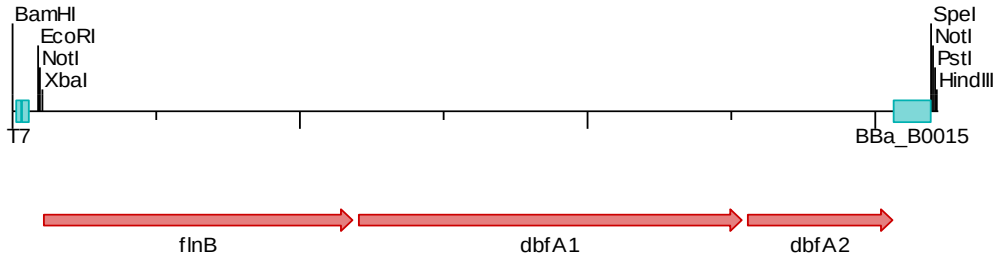
3- GENE CHARACTERISTICS

4.1. FLUORENE

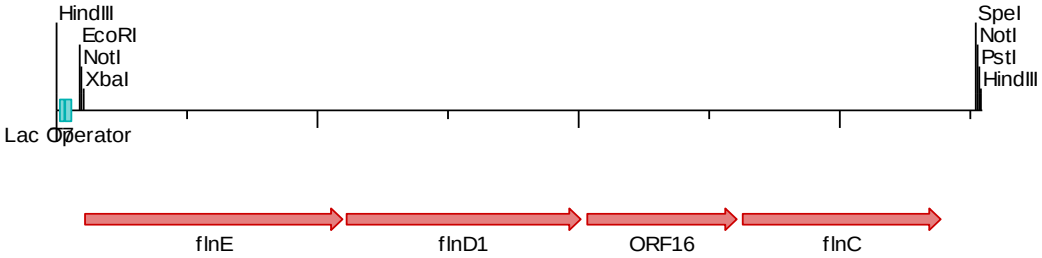
Map of <u>Native</u>Fluorene (Upper pathway) – 11859 bp



Map of SyntheticFluoreneCatabolic Pathway (Insert 1) – 3219 bp:

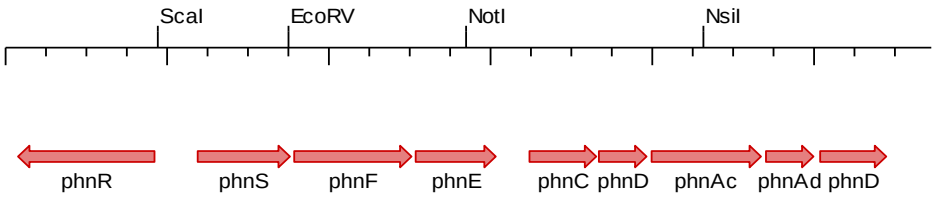


Map of SyntheticFluoreneCatabolic Pathway (Insert 2) – 3545 bp:

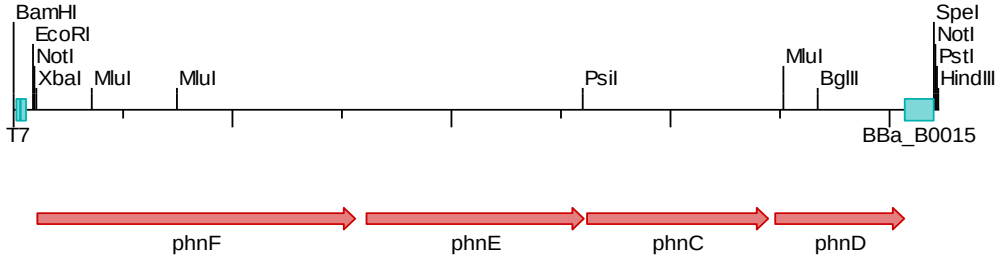


4.1. PHENANTHRENE

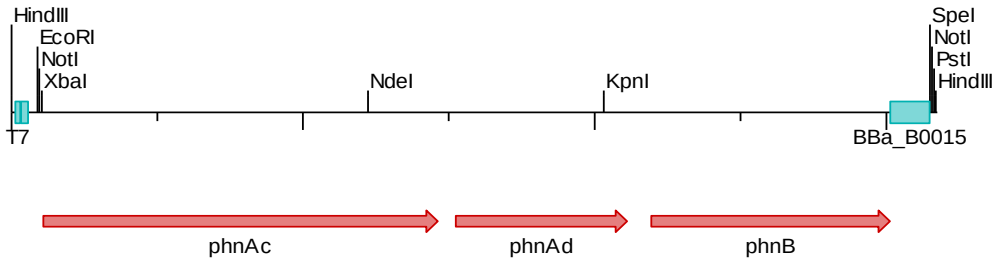
Map of NativePhenanthrene (Upper pathway) – 11451 bp



Map of SyntheticPhenanthreneCatabolic Pathway (Insert 1) – 4227bp:



Map of SyntheticPhenanthrene Catabolic Pathway (Insert 2) – 3174bp:



Plasmid Designation	Description	Size of Insert	Vector
FLUO-Insert 1	Synthetic flnB, dbfA1, dbfA2	3219 bp	pUC57 Ampicillin
FLUO-Insert 2	Synthetic flnE, flnD1, ORF16, flnC	3545 bp	pUC57 Ampicillin
PHE-Insert 1	Synthetic phnF, phnE, phnC, phnD	4227 bp	pUC57 Ampicillin
PHE-Insert 2	Synthetic phnAc, phnAd, phnB	3174 bp	pUC57 Ampicillin

4- DNA PREPARATION

4.2. MATERIALS

a. Reagent grade water
b. The mini-genes were designed by CCA-IGEM-Team 2017.
c. The mini-genes were synthetized by Genscript.
d. The genes were delivered to us lyophilized.

4.3. DNA PREPARATION

The vials containing the lyophilized DNA(~4 µg) were spun down before opening the vials for the first time 16 µL of 0.2µm filtered water was added to the lyophilized powder using a P20 pipet.

After closing the tubes, they were vortexed for 2-3 minutes and were allowed to sit at 60-65°C for 15minutes to resuspend the DNA. The tubes were then spun. Aliquots were taken to start cloning. All stocks are stored at -20°C.

Date of preparation for Fluorene synthetic genes: 20-Jul-2017
Date of preparation for Phenanthrene synthetic genes: 24-July-2017

5- VECTOR PREPARATION

Date: 20-Jul-2017

4.1. MATERIALS

a. Synthetic DNA
   - Synthetic flnB, dbfA1, dbfA2: 3219 bp
   - Synthetic flnE, flnD1, ORF16, flnC: 3545 bp
   - Synthetic phnF, phnE, phnC, phnD: 4227 bp
   - Synthetic phnAc, phnAd, phnB: 3174 bp
b. HindIII restriction enzyme, Thermo Fisher, Cat No. ER0501
c. Restriction digest Buffer R, Thermo Fisher, Cat No. 00267554
d. Antarctic Phosphatase, New England Biolabs Cat No. M0289G
e. Agarose, 100 g, Fisher, Cat No. BP-164-100
f. 50XTAE Electrophoresis Buffer, 1L, (1X: 40 mMTris, 20mM Acetic Acid, 1 mM EDTA), Thermofisher, Cat No. B49
g. Sybr Safe DNA Gel Stain, Invitrogen, Cat No. S33102
h. 100 bp DNA marker, Invitrogen, Cat No. 15628-019
i. 10X Blue Juice DNA loading buffer, Invitrogen, 10816-015
j. Reagent grade water
k. Gel DNA Recovery Kit, Zymo Research, Cat No. D4007
l. 1.5 mL tubes
m. Vortex
n. Ice bucket and ice

4.2. METHODS

Date: 24-Jul-2017

4.2.1. Preparation of recipient vector (insert 1)

a. This insert contains the vector (origin of replication of pUC and the first part of the catabolic pathway under the control of the T7 promoter + inducible lacI +RBS)
b. Digest clone containing synthetic gene containing insert 1 with the restriction enzyme HindIII that cuts only at one site.
c. Restriction Digest Set-up (20µl reaction) in a 1.5 mL tube
d. Add the reagents as described below.
e. Incubate at 37°C for 1 hours
f. Add 2 µl of phosphatase buffer
g. Add 1 µl of phosphatase
h. Incubate at 37°C for 30 minutes
i. Add 2.3 µl of loading buffer at the end of the reaction
j. Load the reaction on a 1% Agarose gel
k. After running the electrophoresis, cut the linearized band.
l. Purify the DNA from the gel using the Zymoclean Gel DNA Recovery Kit

m. Elute the DNA from the Zymoclean column with 12 µl of sterile filtered H2O

Component	Volume	**Final condition**
Reagent grade water	8.0 µl	
10X Buffer R	2.0 µl	1x
Vector-Insert 1	1.0 µl	0.5 µg
HindIII (10U/µl)	1.0 µl	10U

4.2.2. Preparation of insert 2

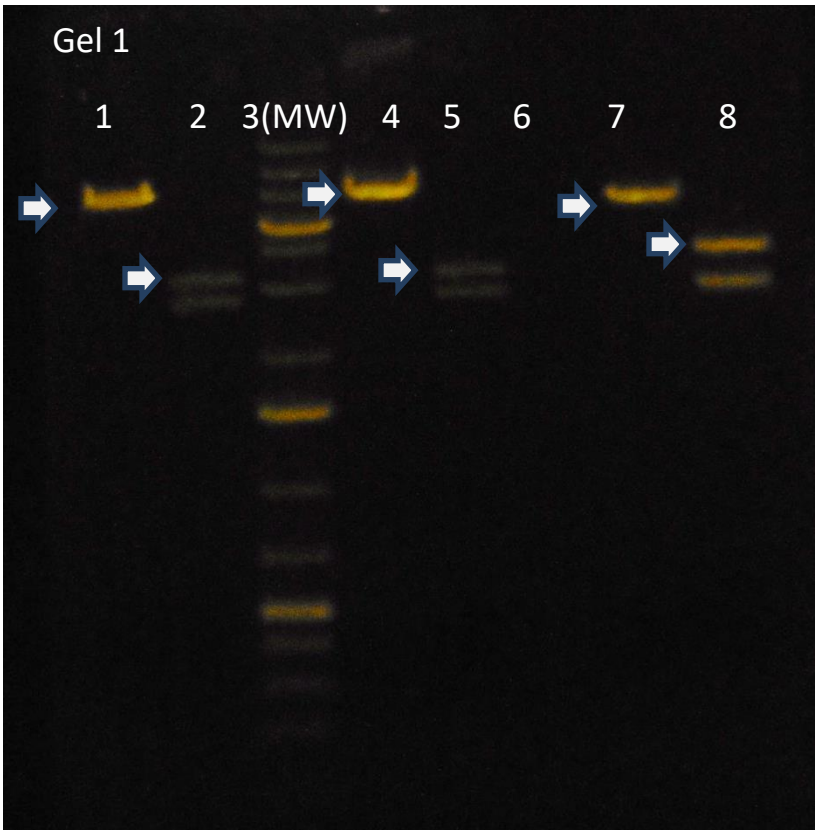
Date: 24-Jul-2017

i. This insert contains the second part of the catabolic pathway under the control of the T7 promoter + inducible lacI +RBS)
ii. Digest plasmid containing synthetic gene designated as insert 2 with the restriction enzyme HindIII that cuts at 2 sites (HindIII sites are flanking the genes)
iii. Set-up restriction digestion (20µl reaction) in a 1.5 mL tube
iv. Turn on water bath at 37°C
v. Add the reagents in the order and with volume described in the table below.
vi. Spin the tube briefly for 15 seconds at 10,000 rpm
vii. Incubate the tube at 37°C for 1 hours
viii. Add 2 µl of loading buffer in the tube at the end of the reaction
ix. Load the digestion reaction on a 1% Agarose gel, TAE
x. After running the electrophoresis for 2 hours at 80V, cut out with a razor blade the linearized band of the desired size.
xi. Purify the DNA from the gel using the Zymoclean Gel DNA Recovery Kit.
xii. Elute the DNA from the Zymoclean column with 12 µl of sterile filtered H2O

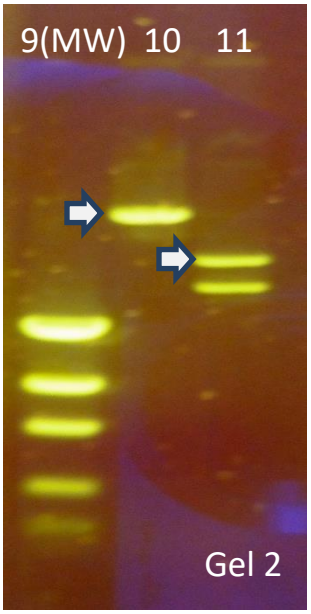
Component	Volume	**Final condition**
Reagent grade water	12.5 µl	
10X Buffer R	1.5 µl	1 X
Preparation of Insert 2	1.0 µl	~0.4 µg
HindIII (10U/µl)	1.0 µl	10U

4.2.3. Gel purification of DNA fragments

Date: 24-Jul-2017



Date: 20-Jul-2017



Gel 1

1) Phenanthrene vector/insert1 digested with HindIII and de-phosphorylated
2) Phenanthrene insert 2 digested with HindIII
3) 1 kb molecular weight ladder
4) Phenanthrene vector/insert1 digested with HindIII and SpeI
5) Phenanthrene insert 2 digested with HindIII and SpeI
6) Empty well
7) Fluorene vector/insert1 digested with HindIII and SpeI
8) Fluorene insert2 digested with HindIII and SpeI

The HindIII-SpeI digest is a back-up just in case the ligation using the HindIII site and dephosphorylation strategy does not work.

Gel 2

9) 1 Kb molecular weight ladder
10) Fluorene vector/insert1 digested with HindIII and de-phosphorylated
11) Fluorene insert 2 digested with HindIII

Arrows indicate the fragments that were cut out and gel-purified and used for ligation.

4.2.4. Ligation

Date: 24-Jul-2017

Materials

a. T4 DNA Ligation Buffer. Invitrogen, Cat No. 46-0114
b. T4 DNA Ligase, Thermo Scientific, Cat No. K1231
c. Reagent grade water, NERL, Cat No. 98555
d. 1.5 mL tube
e. Vortex
f. Pipet and tips
g. Ice bucket and ice

Methods

a. Set ligation as shown below in 1.5 mL tube.
b. The tubes were incubated at room temperature for 1 hour.
c. The tubes were then transferred to ice.

Ligation Condition (with insert)

Component	**Volume (**µl)
Reagent grade water	5.0 µl

10X T4 ligation buffer	1.0 µl
HindIII-Linearized gel purified Vector-Insert 1-dephosphorylated	0.5 µl
HindIII-Linearized -Insert 2	3.0 µl
T4 DNA Ligase (5Weiss/µl)	0.5 µl

Control Ligation Condition (no insert)

Component	**Volume (**µl)
Reagent grade water	8.0 µl
10X T4 ligation buffer	1.0 µl
HindIII-Linearized gel purified Vector-Insert 1-dephosphorylated	0.5 µl
T4 DNA Ligase (5Weiss/µl)	0.5 µl

4.2.5. Transformation

Date: 24-Jul-2017

Materials

a. LB Carbenicillin 50 agar plates, Cat No. Teknova, L1801
b. DH5a competent cells, Invitrogen, Cat 18265-017
c. SOC (Recovery Medium), Lucigen, Cat No. F98226
d. 1.5 mL tube
e. Vortex
f. Pipet and tips

Method

a. Transform ligated DNA into E.coli DH5a chemically competent cells
b. Turn on incubator-shaker at 37°C.
c. Turn on incubator for plates at 37°C.
d. Set up water bath at 42°C.
e. Bring to room temperature S.O.C medium.
f. Bring LB plates supplemented with appropriate antibiotic at room temperature.
g. Thaw competent cells on ice.
h. Aliquots competent cells in as many tubes as needed.
i. Add 10 µl ligation mix to 40 µl competent cells to DNA and swirl gently to mix.

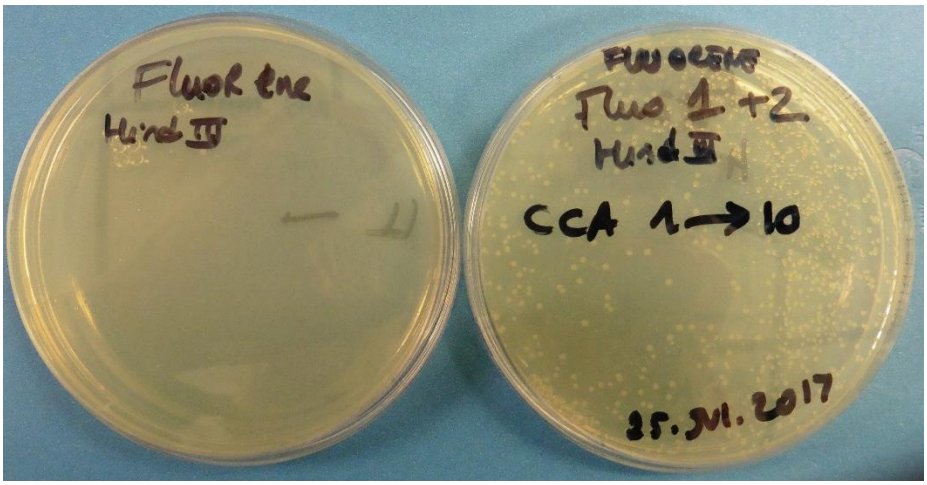
j. Incubate on ice for 20 minutes
k. Heat shock at 42°C in heat block for 30 seconds. Quickly return to ice and let it sit for 2 min.
l. Add 200 µl of 18-25°C SOCmedium and transfer mixture to 15mL Falcon tube
m. Incubate in shaker at 37°C, 225 rpm for 30 min
n. Plate 2 volumes (50 µL and ~100µL) of the mixture onto 2 different plates of LB agar plates supplemented with Carbenicillin 100 µg/mL
o. Incubate plates at 37°C overnight
p. Count colonies and estimate transformation efficiency
q. Initiate cultures in LB with the appropriate antibiotic from individual clones to analyze clones

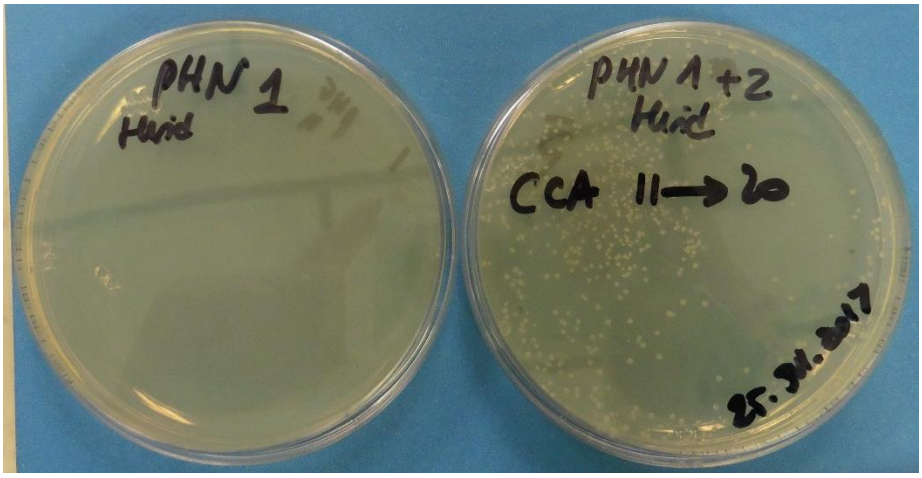
4.2.6. Transformation Results

Date: 25-Jul-2017

Transformation results of mixture plated on 24-July-2017; Readout on 25-Jul-2017

Description	**Number of colonies (50 µL volume plating)**	**Number of clones analyzed by digestion**
Phenanthrene		
Clones derived from Ligation Phenanthrene vector and insert 2 HindIII-Dephos. Synthetic phnF, phnE, phnC, phnD: 4227 bp HindIII- Synthetic phnAc, phnAd, phnB: 3174 bp	>100	10
Clones derived from Ligation Phenanthrene vector alone HindIII-Dephos. Synthetic phnF, phnE, phnC, phnD: 4227 bp	0	
Fluorene		
Clones derived from Ligation Fluorene vector and insert 2 HindIII-Dephos. Synthetic flnB, dbfA1, dbfA2: 3219 bp HindIII- Synthetic flnE, flnD1, ORF16, flnC: 3545 bp	>100	10
Clones derived from Ligation Fluorene vector alone HindIII-Dephos. Synthetic flnB, dbfA1, dbfA2: 3219 bp	0	





4.2.7. Clone Verification

Date: 25-Jul-2017

Materials

a. DNA extraction kit, Zymo Research
b. LB liquid medium
c. Antibiotic stock solution
d. Vortex
e. Pipet and tips
f. 15 mL culture tube
g. Incubator-Shaker
h. 10 mL pipette
i. Pipet aid
j. Vortex
k. Rack
l. Toothpick

Set-up Culture

a. Grow selected number of colonies in 3 mL LB medium supplemented with Ampicillin (100 µg/mL) overnight at 37°C in the incubator/shaker at 220 rpm.
b. Spin down 1.5 mL of culture from each culture and isolate plasmid DNA using the Zymo Research DNA Isolation kit.
c. DNA preparation is resuspended in a final volume of **35 µL** reagent grade water.
d. Store remaining 1mL of culture for glycerol stock preparation of sequence confirmed clones.
e. Check clones by digestion.

Set-up Digestion for Clone Verification –Verification 1

Date: 26-Jul-2017

a. Set up digestion as shown below.
b. This is a 1% gel agarose gel, TAE, prepared with Sybr green dye.
c. Digest clones with EcorRI
d. Set-up restriction digestion (20µl reaction) in a 1.5 mL tube
e. Turn on water bath at 37°C
f. Add the reagents in the order and with volume described in the table below.
g. Spin the tube briefly for 15 seconds at 10,000 rpm
h. Incubate the tube at 37°C for 15 min
i. The reaction already have a loading buffer
j. Load the digestion reaction on a 1% Agarose gel, TAE
k. Set up the power for electrophoresis to 120 V and let it run for ~ 1 hour.

Component	Volume	**Final condition**
Reagent grade water	9.5 µl	
10X BufferFastDigest Green Buffer	1.5 µl	1 X
Plasmid miniprep	3.0 µl	~0.2-0.4 µg
EcoRI	1.0 µl	

Expected Fragments Size for Verification 1

Fluorene:
EcoRI digestion:
If the 2 fragments are in reverse orientation: 6574 bp and 2894 bp
If the 2 fragments are in the same orientation: 6255 bp and 3213 bp

Phenanthrene:
EcoRI digestion:
If the 2 fragments are in reverse orientation: 7211 bp and 2894 bp
If the 2 fragments are in the same orientation: 5884 bp and 4221 bp

Gel Electrophoresis Picture:

Fluorene full length: Clone CCA-1 to CCA-10
Phenanthrene full length: Clone CCA-11 to CCA-20

Results:
Clones CCA-1, CCA-2, and CCA-7 (full length fluorene under the control of an inducible promoter) appear to be correct as the expected size fragments are present after EcoRI digestion.

Clones CCA-12, CCA-15, CCA-17 and CCA-18 (full length phenanthrene under the control of an inducible promoter) appear to be correct as the expected size fragments are present after EcoRI digestion.

Set-up Digestion for Clone Verification –Verification 2
Date: 27-Jul-2017

a. Set up digestion as shown below.
b. This is a 1% gel agarose gel, TAE, prepared with Sybr green dye.
c. Digest clones with PstI
d. Set-up restriction digestion (20µl reaction) in a 1.5 mL tube
e. Turn on water bath at 37°C
f. Add the reagents in the order and with volume described in the table below.

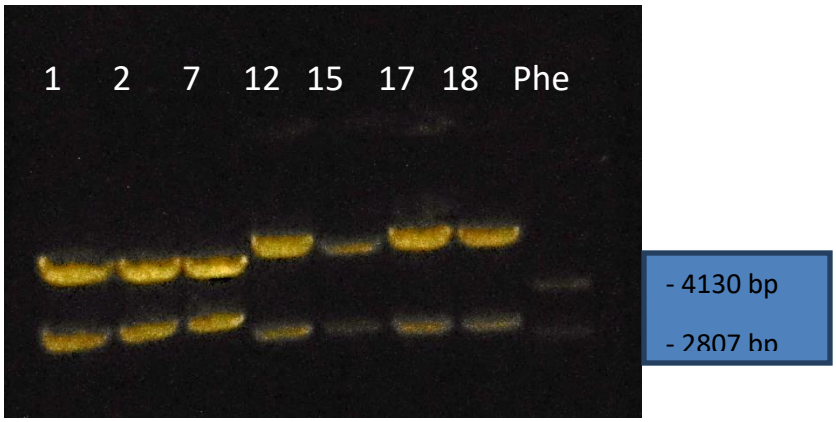
g. Spin the tube briefly for 15 seconds at 10,000 rpm
h. Incubate the tube at 37°C for 1 hour
i. Add 2 µl of loading buffer in the tube at the end of the reaction
j. Load the digestion reaction on a 1% Agarose gel, TAE

Component	Volume	**Final condition**
Reagent grade water	9.5 µl	
10X Buffer FastDigest Green Buffer	1.5 µl	1 X
Plasmid miniprep	3.0 µl	~0.2-0.4 µg
PstI	1.0 µl	

Expected Fragments Size for Verification 2
Fluorene:
PstI digestion:
If the 2 fragments are in reverse orientation: 9546 bp and 12 bp
If the 2 fragments are in the same orientation: 3539 bp and 5929 bp

Phenanthrene:
PstI digestion:
If the 2 fragments are in reverse orientation: 10093 bp and 12 bp
If the 2 fragments are in the same orientation: 3168 bp and 6935 bp

Gel Electrophoresis Picture:



Clone CCA-1 to CCA-7 Fluorene full pathway
Clone CCA-12 to CCA-18 Phenanthrene full pathway
Molecular weight: digestion of phenanthrene upper pathway giving rise to 2 fragments of size 4130 b and 2807 bp.

Results:

Clones CCA-1, CCA-2, and CCA-7 (full length fluorene under the control of an inducible promoter) are the same. These clones seem to indicate that there is a preference in insertion of orientation of the 2 fragments.

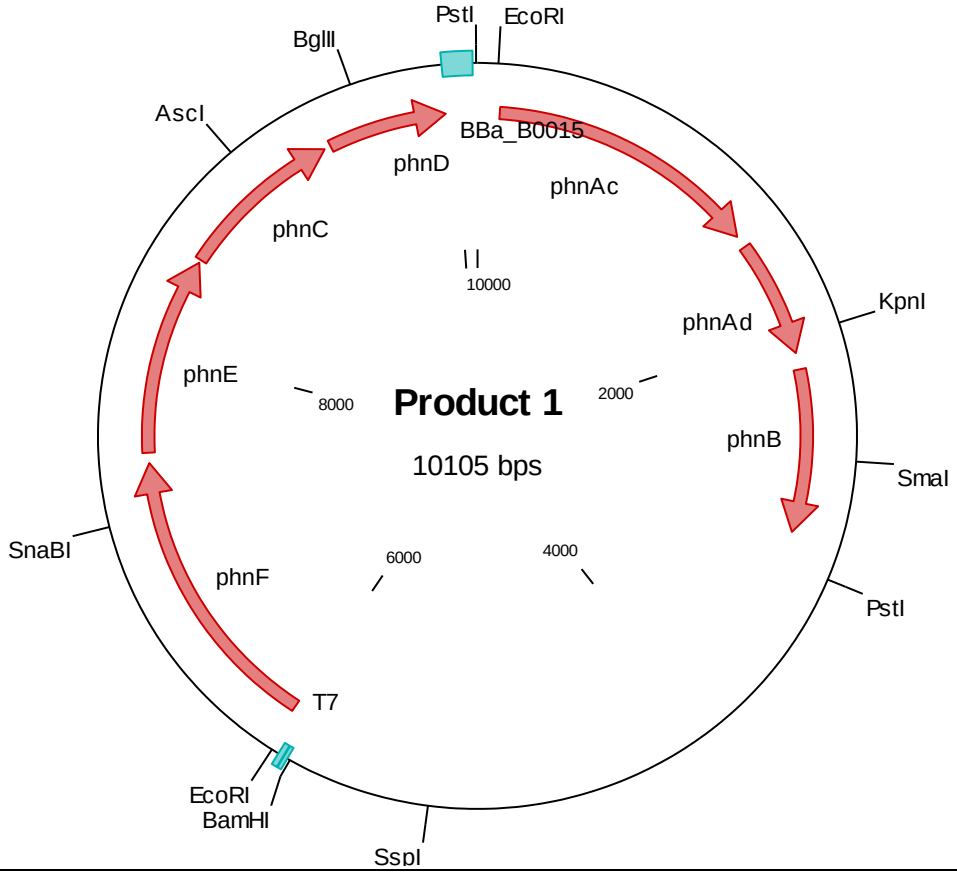
Clones CCA-12, CCA-15, CCA-17 and CCA-18 (full length phenanthrene under the control of an inducible promoter) are the same. These clones seem to indicate that there is a preference in insertion of orientation of the 2 fragments.

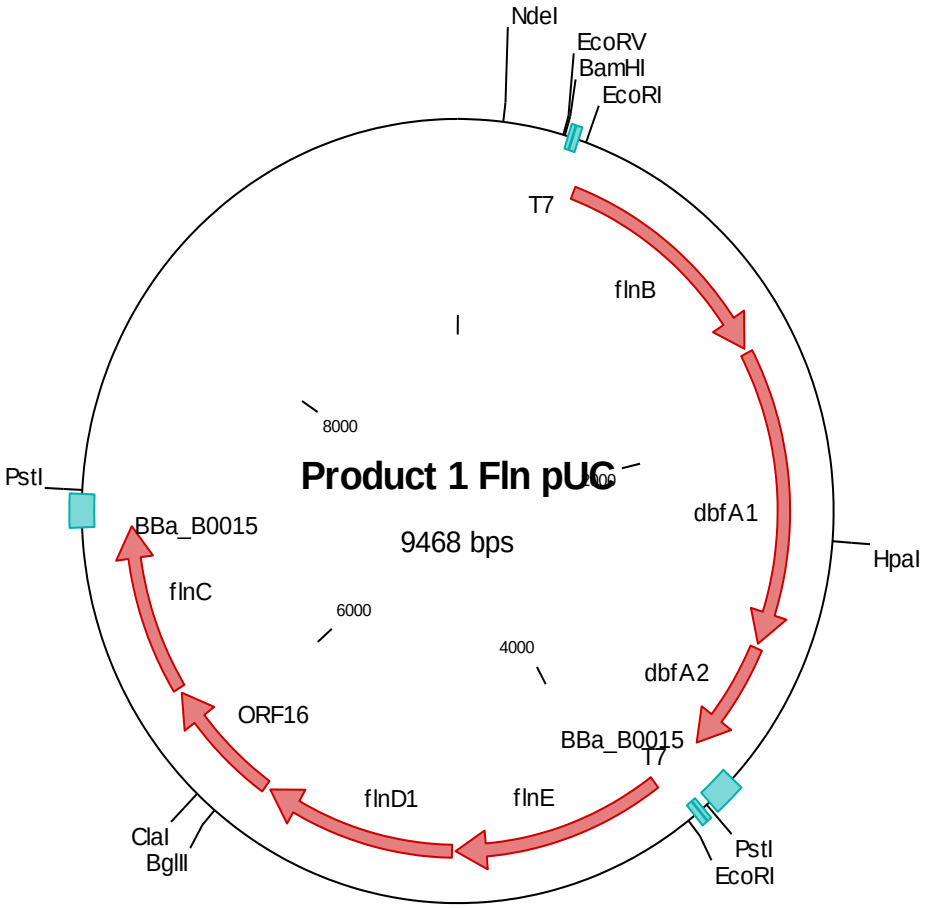
Clone CCA-1 and CCA-12 will be transformed into BL-21 for further analysis of the catabolic gene expression.

Full length Synthetic fluorene catabolic pathway =
Fragment Synthetic flnB, dbfA1, dbfA2: 3219 bp + fragment Synthetic flnE, flnD1, ORF16, flnC: 3545 bp

Full length Synthetic Phenanthrene catabolic pathway =
Synthetic phnF, phnE, phnC, phnD: 4227 bp + Synthetic phnAc, phnAd, phnB: 3174 bp

Plasmid Maps:




NdeI
EcoRV
BamHI
EcoRI
T7
flnB
8000
Product 1 Fln pUC
9468 bps
dbfA1
HpaI
PstI
BBa_B0015
flnC
6000
4000
dbfA2
ORF16
BBa_B0015
T7
flnD1
flnE
ClaI
BglII
PstI
EcoRI

6- GENERATION OF CONSTRUCTS BL-21

Date: Date: 27-Jul-2017

6.1. MATERIALS

a. DNA samples
b. Transformation efficiency DNA, pUC 19, 10 pg/ µL, Lucigen, Cat No. F92078-1
c. LB Carbenicillin 50 agar plates, Cat No. Teknova, L1801
d. E coli BL21 DE3, Life Technology, Cat No. 60106-1
e. SOC (Recovery Medium), Lucigen, Cat No. F98226
f. 15 mL culture tube
g. Vortex
h. Tooth pick
i. Incubator shaker
j. 42°C Water bath
k. Ice and ice bucket
l. Pipet

6.2. METHODS

a. Transform 1 µL of DNA mini-preparation into E coli BL21 DE3 chemically competent cells
b. Transform 1 µL of DNA of pUC19 as transformation efficiency control
c. Turn on incubator-shaker at 37°C.
d. Turn on incubator for plates at 37°C.
e. Set up water bathat 42°C.
f. Bring to room temperature S.O.C medium.
g. Bring LB plates supplemented with appropriate antibiotic at room temperature.
h. Thaw competent cells on ice.
i. Aliquots competent cells in as many tubes as needed.
j. Add 1.0 µl DNA preparation to 40 µl competent cells to DNA and swirl gently to mix
k. Incubate on ice for 20 minutes
l. Heat shock at 42°C in heat block for 30 seconds. Quickly return to ice and let it sit for 2 min.
m. Add 200 µl of 18-25°C SOCmedium and transfer mixture to 15mL Falcon tube
n. Incubate in shaker at 37°C, 225 rpm for 30 min
o. Plate 2 volumes (50 µL and ~100µL) of the mixture onto 2 different plates of LB agar plates supplemented with Carbenicillin 100 µg/mL
p. Incubate plates at 37°C overnight
q. Count colonies and estimate transformation efficiency
r. Initiate cultures in LB with the appropriate antibiotic from individual clones to analyze clones by digestion

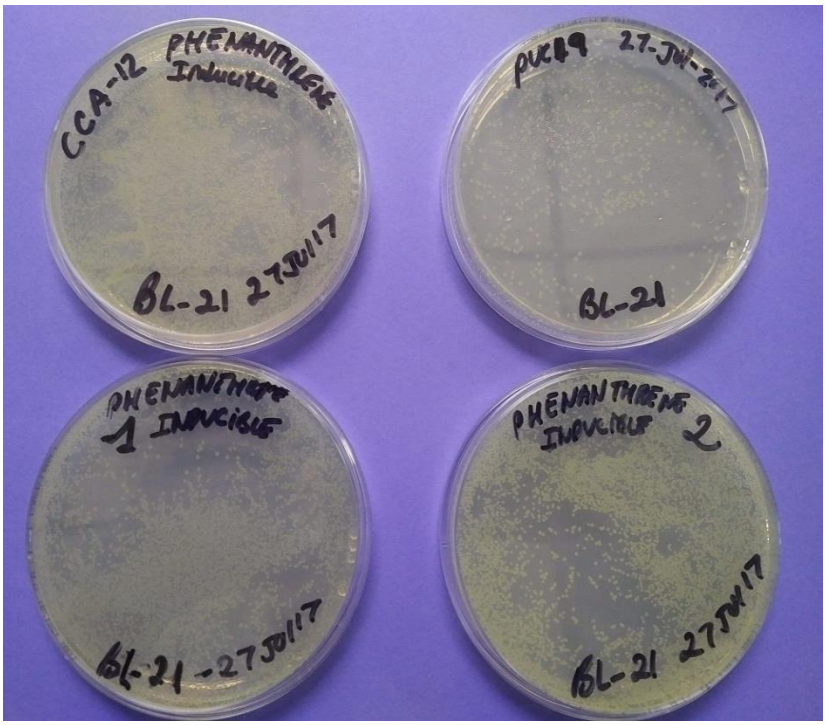


Figure. LB-Carbenicillin plates with E.coli BL-21 cells containing plasmids CCA-12 (full phenanthrene catabolic pathway), Phenanthrene-1, and Phenanthrene-2. pUC19 was also transformed as a control.

Full length Synthetic Phenanthrene catabolic pathway = Synthetic phnF, phnE, phnC, phnD: 4227 bp + Synthetic phnAc, phnAd, phnB: 3174 bp cloned in pUC57

Phenanthrene-1= Synthetic phnF, phnE, phnC, phnD: 4227 bp cloned in pUC57

Phenanthrene-2= Synthetic phnAc, phnAd, phnB: 3174 bp cloned in pUC 57

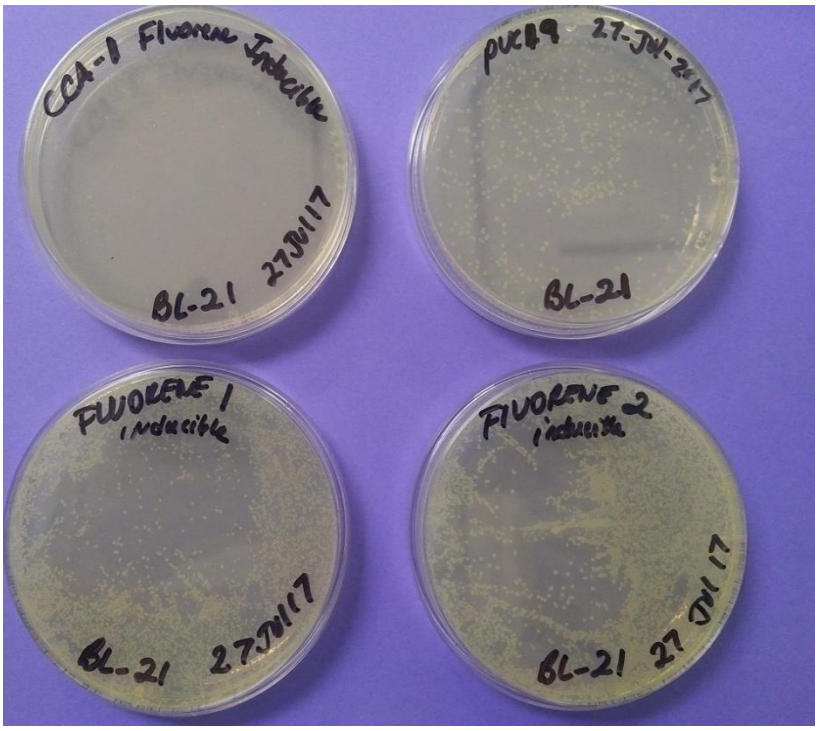


Figure. LB-Carbenicillin plates with E.coli BL-21 cells containing plasmids CCA-12 (full fluorine catabolic pathway), Fluorene-1 and Fluorene-2.

pUC19 was also transformed as a control.

Full length Synthetic fluorene catabolic pathway = Synthetic flnB, dbfA1, dbfA2: 3219 bp + Synthetic flnE, flnD1, ORF16, flnC: 3545 bp

Fluorene-1= Synthetic flnB, dbfA1, dbfA2: 3219 bp: 4227 bp cloned in pUC57

Fluorene-2= Synthetic flnE, flnD1, ORF16, flnC: 3545 bp cloned in pUC 57

Note: The bacteria with the full fluorene pathway are tiny. They are growing very slowly.

7- GLYCEROL STOCK

Date: 29-Jul-2017

Materials

a. Glycerol
b. LB liquid medium
c. Antibiotic stock solution
d. 15 mL culture tube
e. Incubator-Shaker
f. 10 mL pipette
g. Pipet aid
h. Vortex
i. Rack
j. Toothpick

Set-up Culture and Prepare Glycerol Stocks

a. Grow selected clones that have been checked (so they are correct) in 3 mL LB medium supplemented with Ampicillin (100 µg/mL) overnight at 37°C in the incubator/shaker at 220 rpm.
b. Prepare LB medium with 40% glycerol and add 0.5 mL to a cryogenic vial
c. Add 0.5 mL of culture sample to be stored
d. Gently vortex the cryogenic vial to ensure the culture and glycerol is well mixed
e. Label tube with date and identifier
f. Organize in a freezer box and label box
g. Prepare excel spreadsheet with all information
h. Store freezer box at -80°C

Glycerol Stock	Date of Glycerol Stock	Clone	Description	Cell Description	Selection Marker	Vector
CCA-1000	29-Jul-17	CCA-1	Full Synthetic catabolic pathway Fluorene Inducible promoter	E.coli DH5a	Ampicillin	pUC57
CCA-1001	29-Jul-17	CCA-12	Full Synthetic catabolic pathwayPhenanthrene Inducible promoter	E.coli DH5a	Ampicillin	pUC57
CCA-1002	29-Jul-17	CCA-1	Full Synthetic catabolic pathway Fluorene Inducible promoter	E.coli BL-21	Ampicillin	pUC57
CCA-1003	29-Jul-17	CCA-12	Full Synthetic catabolic pathway Phenanthrene Inducible promoter	E.coli BL-21	Ampicillin	pUC57
CCA-1004	29-Jul-17	Synthetic Fluorene-1	Synthetic flnB, dbfA1, dbfA2 Inducible promoter	E.coli BL-21	Ampicillin	pUC57
CCA-1005	29-Jul-17	Synthetic Fluorene-2	Synthetic flnE, flnD1, ORF16, flnC Inducible promoter	E.coli BL-21	Ampicillin	pUC57
CCA-1006	29-Jul-17	Synthetic Phenanthrene 1	Synthetic phnF, phnE, phnC, phnD Inducible promoter	E.coli BL-21	Ampicillin	pUC57
CCA-1007	29-Jul-17	Synthetic Phenanthrene 2	Synthetic phnAc, phnAd, phnB Inducible promoter	E.coli BL-21	Ampicillin	pUC57
CCA-1008	29-Jul-17	pUC19		E.coli BL-21	Ampicillin	pUC19