Laboratory Records: Cloning of Synthetic Catabolic Pathways of Fluorene and Phenanthrene Under the Control of Constitutive Promoters

Table of Contents

1- PURPOSE

-Cloning of fluorene and phenanthrene catabolic pathways under the control of constitutive promoters.
-Testing of 3 different constitutive promoters driving the catabolic pathways in E.coli and in other bacteria selected for gene augmentation.

2- CLONING STRATEGY

2.1. BACKGROUND

The catabolic pathway for fluorene and phenanthrene were synthetized as polycistronic operons with the codon optimized for expression in *E.coli*.
The polycistronic catabolic pathways for fluorene and phenanthrene were split into 2 parts for several reasons, F1 and F2 for fluorene and P1 and P2 for phenanthrene:
(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 in single bacteria.

We have tested the full pathway P1_P2 and F1_F2 under the control of the T7LacZ inducible promoter. From that experiment, we have learned that the expression of the enzymes involved in the fluorene catabolic pathway may be slightly toxic as illustrated by slow bacterial growth. Therefore, the fluorene pathway will be cloned into a low copy number plasmid (origin of replication: p15a). The expression of the enzymes involved in the phenanthrene catabolic pathway did not appear to alter bacterial growth when the pathway is under the control of the T7LacZ inducible promoter. Therefore, the phenanthrene catabolic pathway will be cloned into pSB1C3 (high copy number plasmid). The 2 plasmids are compatible in E.coli, so they can be transformed into the same bacterial strain for the degradation of both fluorene and phenanthrene

P1: Synthetic phnF, phnE, phnC, phnD
P2: Synthetic phnAc, phnAd, phnB

F1: Synthetic flnB, dbfA1, dbfA2
F2: Synthetic flnE, flnD1, ORF16, flnC

2.2. STRATEGY

<u>Phenanthrene Catabolic Pathway:</u>
Step 1: Design 3 promoter area containing the prefix sequence, followed by one of the 3 constitutive promoters of part BBa_J23100 or BBa_J23101 or BBa_J23110 followed by a Ribosome Binding Site of part BBa_B0034, followed by the suffix sequence.
Step 2: Order sequence at IDT to be cloned into pIDT_kanamycin vector to facilitate subsequent cloning into vectors with different antibiotic resistance genes (=pIDT is a pUC plasmid)
Step 3: Prepare, ligate and transform the following Linearized DNA fragments to obtain each pathway P1 and P2 under the control of 3 different constitutive promoters on two separate plasmids:

Linearized promoter area fragments as EcoRI/SpeI
Linearized each of the pathway fragment (P1 and P2) as XbaI/PstI
Linearized vector pSB1C3 as EcoRI/PstI

Step 4: Check clones by digestion for insertion of correct fragments
Step 5: Prepare, ligate, and transform the following Linearized to obtain pathway P1 and P2 under the control of 3 different constitutive promoters on the same plasmid:

Linearized promoter area + P1 fragments + vector pSB1C3 as SpeI/PstI
Linearized promoter area + P2 fragments as XbaI/PstI

Step 6: Check clones by digestion and sequencing for insertion of correct fragments
Step 7: Transform correct clones into E.coli BL-21 for gene expression and growth in LB, minimal medium supplemented with various sources of carbons (glucose and/or phenanthrene).

Fluorene Catabolic Pathway:

Step 1: Use the promoter areas designed above [3 promoter area containing the prefix sequence, followed by one of the 3 constitutive promoters of part BBa_J23100 or BBa_J23101 or BBa_J23110 followed by a Ribosome Binding Site of part BBa_B0034, followed by the suffix sequence].
Step 2: Prepare, ligate and transform the following Linearized DNA fragments to obtain each pathway F1 and F2 under the control of 3 different constitutive promoters on two separate plasmids:

Linearized promoter area fragments as EcoRI/SpeI
Linearized each of the pathway fragment (F1 and F2) as XbaI/PstI
Linearized vector pSB1C3 as EcoRI/PstI

Step 3: Check clones by digestion for insertion of correct fragments
Step 4: Prepare, ligate, and transform the following Linearized to obtain pathway F1 and F2 under the control of 3 different constitutive promoters on the same plasmid:

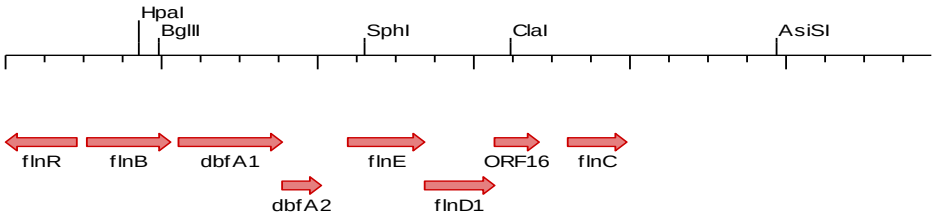
Linearized promoter area + F1 fragments as EcoRI/SpeI
Linearized promoter area + F2 fragments as XbaI/PstI
Linearized vector p15a as EcoRI/PstI [pSB3T5 Tetracycline]

Step 5: Check clones by digestion and sequencing for insertion of correct fragments
Step 6: Transform correct clones into E.coli BL-21 for gene expression and growth in LB, minimal medium supplemented with various sources of carbons (glucose and/or phenanthrene).

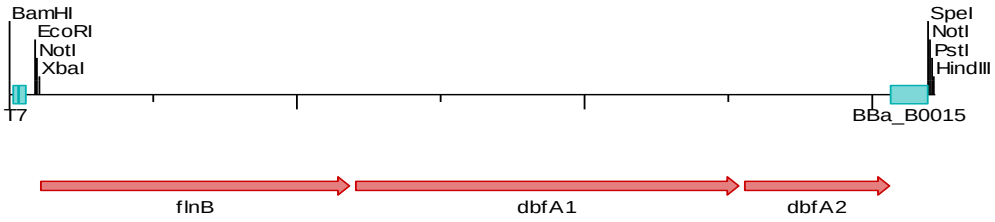
3- GENE AND PROMOTER AREAS CHARACTERISTICS

3.1. FLUORENE

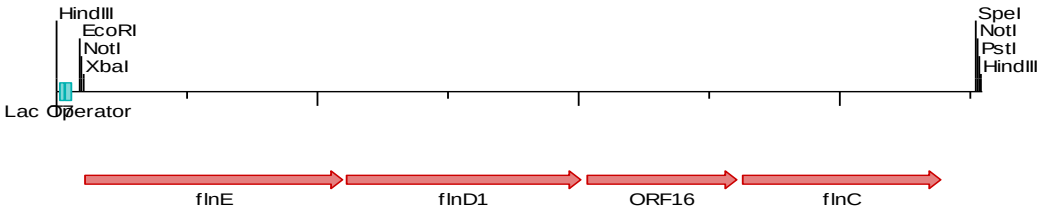
Map of Native Fluorene (Upper pathway) – 11859 bp



Map of Synthetic Fluorene Catabolic Pathway (Insert 1) – 3219 bp:

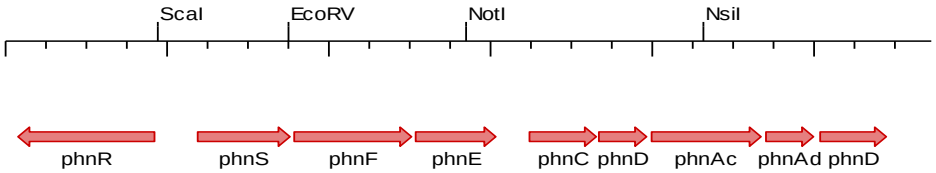


Map of <u>Synthetic</u> Fluorene Catabolic Pathway (Insert 2) – 3545 bp:

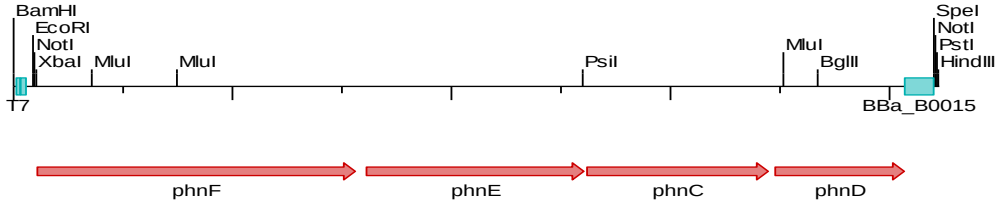


3.2. PHENANTHRENE

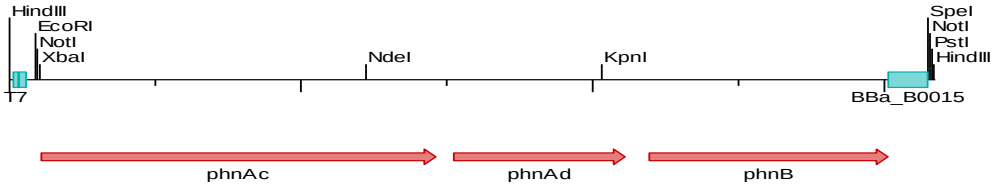
Map of <u>Native</u> Phenanthrene (Upper pathway) – 11451 bp



Map of <u>Synthetic</u> Phenanthrene Catabolic Pathway (Insert 1) – 4227bp:



Map of <u>Synthetic</u> Phenanthrene Catabolic Pathway (Insert 2) – 3174bp:



Plasmid Designation	Description	Size of Insert	Vector
FLUORENE-Insert 1=F1	Synthetic flnB, dbfA1, dbfA2	3219 bp	pUC57 Ampicillin
FLUORENE-Insert 2=F2	Synthetic flnE, flnD1, ORF16, flnC	3545 bp	pUC57 Ampicillin
PHENANTHRENE-Insert 1=P1	Synthetic phnF, phnE, phnC, phnD	4227 bp	pUC57 Ampicillin
PHENANTHRENE-Insert 2=P1	Synthetic phnAc, phnAd, phnB	3174 bp	pUC57 Ampicillin

3.3. DESIGN OF PROMOTER AREAS

3.3.1. **Assembly using** Constitutive_Promoter_BBa_J23100

>BBa_J23100 Part-only sequence (35 bp)
ttgacggctagctcagtcctaggtacagtgctagc

Sequence:
>BBa_J23100_RBS
GAATTCGCGGCCGCTTCTAGAttgacggctagctcagtcctaggtacagtgctagcaaagaggagaaaACTAGTAGCGGCCGCTGCAG

Description:
EcorI NotI XbaI Constitutive_Promoter_BBa_J23100 RBS_BBa_B0034 SpeI NotI PstI

3.3.2. **Assembly using** Constitutive_Promoter_BBa_J23101

>BBa_J23101 Part-only sequence (35 bp)
tttacagctagctcagtcctaggtattatgctagc

Sequence:
>BBa_J23101_RBS
GAATTCGCGGCCGCTTCTAGAtttacagctagctcagtcctaggtattatgctagc
aaagaggagaaaACTAGTAGCGGCCGCTGCAG

Description:
EcorI NotI XbaI Constitutive_Promoter_BBa_J23101 RBS_BBa_B0034 SpeI NotI PstI

3.3.3. **Assembly using** Constitutive_Promoter_BBa_J23110

```
>BBa_J23101 Part-only sequence (35 bp)
tttacagctagctcagtcctaggtattatgctagc

Sequence:
>BBa_J23110_RBS
GAATTCGCGGCCGCTTCTAGAtttacggctagctcagtcctaggtacaatgctagcaaagaggagaaaACTAGTAGCGGCCGCT
GCAG

Description:
EcorI NotI XbaI Constitutive_Promoter_BBa_J23110 RBS_BBa_B0034 SpeI NotI PstI
```

3.3.4. Alignment of 3 Promoter Areas

```
CLUSTAL O(1.2.4) multiple sequence alignment
BBa_J23101_RBS      GAATTCGCGGCCGCTTCTAGAtttacagctagctcagtcctaggtattatgctagcaaag
BBa_J23100_RBS      GAATTCGCGGCCGCTTCTAGAttgacggctagctcagtcctaggtacagtgctagcaaag
BBa_J23110_RBS      GAATTCGCGGCCGCTTCTAGAtttacggctagctcagtcctaggtacaatgctagcaaag
                    ********************** ** ******************   ***********

BBa_J23101_RBS      aggagaaaACTAGTAGCGGCCGCTGCAG
BBa_J23100_RBS      aggagaaaACTAGTAGCGGCCGCTGCAG
BBa_J23110_RBS      aggagaaaACTAGTAGCGGCCGCTGCAG
                    ***************************
```

3.3.5. Promoter Areas Designation

Plasmid Designation	Description	Size of Insert	Vector
100	BBa_J23100_RBS	88 bp	pIDT_Kanamycin
101	BBa_J23101_RBS	88 bp	pIDT_Kanamycin
110	BBa_J23110_RBS	88 bp	pIDT_Kanamycin

4- DNA SOURCE

4.1. MATERIALS

Promoters (100, 101, 110):

a. Reagent grade water
b. The promoterareas were designed by CCA-IGEM-Team 2017.
c. The promoter areas were synthetized by IDT.
d. The synthetic promoter areas were delivered to us lyophilized.

Polycistronic codons (F1, F2, P1, P2):

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.

4.2. 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 promoter synthetic areas: 1-Aug-2017

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

5- CLONING OF INDIVIDUAL POLYCISTRONIC F1, F2 (FLUORENE) AND P1, P2 (PHENANTHRENE) BEHIND CONSTITUTIVE PROMOTERS

5.1. MATERIALS

a. Synthetic DNA [Fluorene F1, F2] and [Phenanthrene P1 and P2]
   - Synthetic F1 : flnB, dbfA1, dbfA2: 3219 bp
   - Synthetic F2: flnE, flnD1, ORF16, flnC: 3545 bp
   - Synthetic P1: phnF, phnE, phnC, phnD: 4227 bp
   - Synthetic P2: phnAc, phnAd, phnB: 3174 bp

b. Synthetic DNA [Promoter areas 100, 101, 110]
   - Synthetic Promoter 100: 88 bp
   - Synthetic Promoter 101: 88 bp
   - Synthetic Promoter 110: 88 bp

c. Agarose, 100 g, Fisher, Cat No. BP-164-100
d. 50XTAE Electrophoresis Buffer, 1L, (1X: 40 mMTris, 20mM Acetic Acid, 1 mM EDTA), Thermofisher, Cat No. B49
e. Sybr Safe DNA Gel Stain, Invitrogen, Cat No. S33102
f. 1kb plus DNA ladder DNA marker, Thermo Scientific, Cat No. SM1334
g. 10X Blue Juice DNA loading buffer, Invitrogen, 10816-015
h. Reagent grade water
i. Gel DNA Recovery Kit, Zymo Research, Cat No. D4007
j. 1.5 mL tubes
k. Vortex
l. Ice bucket and ice
m. Cryo box for restriction enzyme
n. P1000 and P200 with corresponding tips

5.2. METHODS

5.2.1. Preparation of promoter areas with RBS

Date: 1-Aug-2017

a. The promoters/RBS regions were designed by the CCA_IGEM team and synthetized by IDT.
b. For the writing on plates, the designations for the promoters + RBS were as follows:
    1) BBa_J23100 + RBS =100
    2) BBa_J23101 + RBS =101
    3) BBa_J23110 + RBS =110
c. Double Digestion of promoter + RBS region with restriction enzymes EcoRI and SpeI
d. Restriction Digest Set-up (20µl reaction) in a 1.5 mL tube
e. Add the reagents as described below.
f. Incubate the tube at 37°C for 15 min
g. Note: there is no need to add a loading buffer because the digestion buffer already includes it.
h. Load the reaction on a 1.5 % Agarose gel, TAE
i. After running the electrophoresis for 1 hour at 80V, cut the linearized band.
j. Purify the DNA from the gel using the Zymoclean Gel DNA Recovery Kit
k. Elute the DNA from the Zymoclean column with 12 µl of sterile filtered H2O

Component	Volume	**Final condition**
Reagent grade water	12.0 µl	
10X Buffer	2.0 µl	1x
Promoter +RBS region	4.0µl	~ 1 µg Note: the fragment to recover is of small size (<88bp), so needs more DNA for a reasonable recovery from the gel
EcoRI	1.0 µl	
SpeI	1.0 µl	

5.2.2. Preparation of insert F1, F2, P1, and P2

Date: 31-Jul-2017

a. The following polycistronic sequences are cloned behind the 3 different promoter regions:
    1. For fluorene catabolic pathway
    a. FLUO-Insert 1 or F1 [Synthetic flnB, dbfA1, dbfA2]
    b. FLUO-Insert 2 or F2 [Synthetic flnE, flnD1, ORF16, flnC]
    2. For phenanthrene catabolic pathway
    a. PHE-Insert 1 or P1 [Synthetic phnF, phnE, phnC, phnD]
    b. PHE-Insert 2 or P2 [Synthetic phnAc, phnAd, phnB]
b. The fragments are digested with 2 enzymes: XbaI and Pst I

c. Digest plasmid containing synthetic gene designated F1, F2, P1, and P2 with the restriction enzyme XbaI and PstI
d. Set-up restriction digestion (15 µl 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. Note: there is no need to add a loading buffer because the digestion buffer already has it.
j. Load the digestion reaction on a 1% Agarose gel, TAE
k. After running the electrophoresis for 2 hours at 80V, cut out with a razor blade the linearized band of the desired size.
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	10.0µl	
10X Buffer	1.5 µl	1 X
Preparation of Insert F1, F2, P1 and P2	1.5µl	~0.4 µg
XbaI	1.0 µl	
PstI	1.0 µl	

5.2.3. Preparation of recipient vector

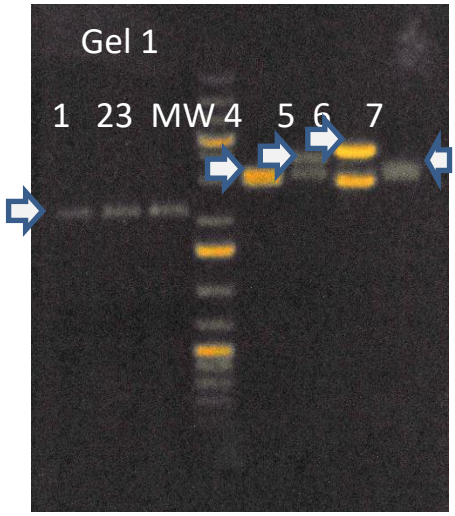
a. The recipient vector is pSB1C3 (carrying the chloramphenicol resistance gene)
b. Double Digestion of promoter + RBS region with EcoRI and PstI
c. Restriction Digest Set-up (15 µl reaction) in 3 X 1.5 mL tube
d. Add the reagents as described below.
e. Incubate the tube at 37°C for 15 min
f. Note: there is no need to add a loading buffer because the digestion buffer already has it.
g. Load the reaction on a 1.0 % Agarose gel
h. After running the electrophoresis for 2 hours at 80V, cut the linearized band.
i. Purify the DNA from the gel using the Zymoclean Gel DNA Recovery Kit
j. Elute the DNA from the Zymoclean column with 12 µl of sterile filtered H2O

Component	Volume	**Final condition**
Reagent grade water	10.0 µl	

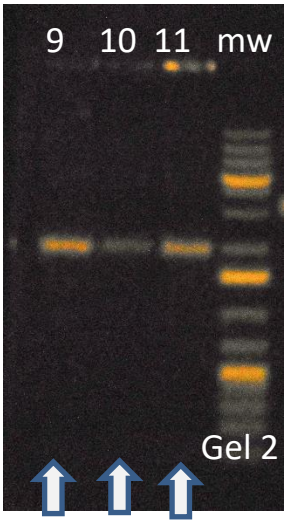
10X Buffer	1.5 µl	1x
pSB1C3	1.5 µl	~ 0.2 µg
EcoRI	1.0 µl	
SpeI	1.0 µl	

5.2.3. Gel purification of DNA fragments

Date: 31-Jul-2017



Date: 1-Aug-2017



Gel 1

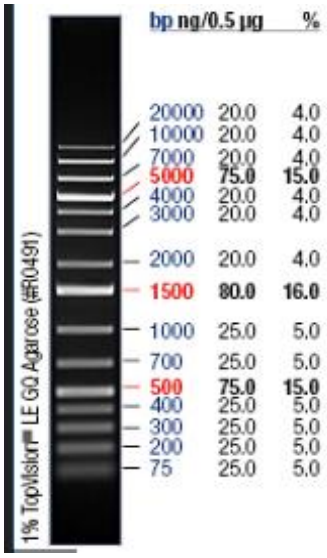
1) pSB1C3 vector digested with EcoR1 and Pst 1
2) pSB1C3 vector digested with EcoR1 and Pst 1
3) pSB1C3 vector digested with EcoR1 and Pst 1
   (We did 3 digestions in parallel to prepare a large stock of this linearized vector as it is needed for multiple cloning)
4) 1 kb molecular weight ladder
5) Fluorene insert 1 XbaI/PstI
6) Fluorene insert 2 XbaI/PstI
7) Phenanthrene insert 1 XbaI/PstI
8) Phenanthrene insert 2 XbaI/PstI

Gel 2

9) Promoter BBa_J23100digested with EcorI and SpeI
10) Promoter BBa_J23101 digested with EcorI and SpeI
11) Promoter BBa_J23110 digested with EcorI and SpeI
12) 1 Kb molecular weight ladder

Arrows indicate the fragments that were cut out and gel-purified and used for ligation. Fragments were visible on the gels but not necessarily on the picture.

DNA Ladder



5.2.4. Ligation

Date: 1-Aug-2017 and 2-Aug-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	4.5 µl
10X T4 ligation buffer	1.0 µl
EcoR1-Pst1 Linearized - pSB1C3	0.5 µl
EcoR1/SpeI - Linearized –promoter BBa_J23100 BBa_J23101 BBa_J23110	2.0 µl
XbaI/Pst1 - Linearized -Insert [Catabolic pathway) Fluorene 1 Or Fluorene 2 Or Phenanthrene 1 Or Phenanthrene 2	1.5 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
EcorR1-Pst1 pSB1C3	0.5 µl

T4 DNA Ligase (5Weiss/µl)	0.5 µl

5.2.5. Transformation

Date: 1-Aug-2017 and 2-Aug-2017

Materials

a. LB Chloramphenicol 34 µg/mL agar plates, Cat No. Teknova, L1017
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
g. Ice bucket and ice
h. Water bath (42°C)
i. Incubator (37°C)

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 (chloramphenicol 34 µg/mL) at room temperature.
g. Thaw competent cells on ice.
h. Aliquots competent cells in as many tubes as needed.
i. Add 5 µl ligation mix to 50 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 SOC medium 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 Chloramphenicol 34 µg/mL
o. Incubate plates at 37°C overnight
p. Count colonies
q. Initiate cultures in LB with the appropriate antibiotic from individual clones to analyze clones

5.3. RESULTS

5.3.1. Transformation Results

Date: 25-Jul-2017

Transformation results of mixture plated on 1-Aug-2017 and 2-Aug-2017; Readout on 2-Aug-2017 and 3-Aug-2017

Description	**Number of colonies (50 µL volume plating)**	**Number of clones analyzed by digestion**
Phenanthrene		
Vector (2080 bp, EcorI/PstI) + Promoter BBa_J23100/RBS_BBa_B0034 (68 bp, EcoRI/SpeI) + PhenanthreneSynthetic P1: phnF, phnE, phnC, phnD_Ter_BBa_B0015: (4115 bp, XbaI/PstI)		2
Vector (2080 bp, EcorI/PstI) + Promoter BBa_J23101/RBS_BBa_B0034 (68 bp, EcoRI/SpeI) + Phenanthrene Synthetic P1: phnF, phnE, phnC, phnD_Ter_BBa_B0015: (4115 bp, XbaI/PstI)	>300	2
Vector (2080 bp, EcorI/PstI) + Promoter BBa_J23110/RBS_BBa_B0034 (68 bp, EcoRI/SpeI) + Phenanthrene Synthetic P1: phnF, phnE, phnC, phnD_Ter_BBa_B0015: (4115 bp, XbaI/PstI)	>300	2
Vector (2080 bp, EcorI/PstI) + Promoter BBa_J23100/RBS_BBa_B0034 (68 bp, EcoRI/SpeI)+ Phenanthrene Synthetic P2: phnAc, phnAd, phnB_Ter_BBa_B0015: (3062 bp, XbaI/PstI)	>300	2
Vector (2080 bp, EcorI/PstI) + Promoter BBa_J23101/RBS_BBa_B0034 (68 bp, EcoRI/SpeI) +Phenanthrene Synthetic P2: phnAc, phnAd, phnB_Ter_BBa_B0015: (3062 bp, XbaI/PstI)	>300	2
Vector (2080 bp, EcorI/PstI) + Promoter BBa_J23110/RBS_BBa_B0034 (68 bp, EcoRI/SpeI)+ PhenanthreneSynthetic P2: phnAc, phnAd, phnB_Ter_BBa_B0015: (3062 bp, XbaI/PstI)	>300	2
Fluorene		
Vector (2080 bp, EcorI/PstI) + Promoter BBa_J23100/RBS_BBa_B0034 (68 bp, EcoRI/SpeI)+ Fluorene Synthetic F1: flnB, dbfA1, dbfA2_Ter_BBa_B0015: (3107 bp, XbaI/PstI)	>300	2
Vector (2080 bp, EcorI/PstI) + Promoter BBa_J23101 (68 bp, EcoRI/SpeI)+ Fluorene Synthetic F1: flnB, dbfA1, dbfA2_Ter_BBa_B0015: (3107 bp, XbaI/PstI)	>300	2

Vector (2080 bp, EcorI/PstI) + Promoter BBa_J23110/RBS_BBa_B0034 (68 bp, EcoRI/SpeI)+ Fluorene Synthetic F1: flnB, dbfA1, dbfA2_Ter_BBa_B0015: (3107 bp, XbaI/PstI)	>300	2
Vector (2080 bp, EcorI/Pst)) + Promoter BBa_J23100/RBS_BBa_B0034 (68 bp, EcoRI/SpeI)+ Fluorene Synthetic F2: flnE, flnD1, ORF16, flnC_Ter_BBa_B0015: (3433 bp, XbaI/PstI)	>300	2
Vector (2080 bp, EcorI/PstI) + Promoter BBa_J23101/RBS_BBa_B0034 (68 bp, EcoRI/SpeI)+ Fluorene Synthetic F2: flnE, flnD1, ORF16, flnC_Ter_BBa_B0015: (3433 bp, XbaI/PstI)	>300	2
Vector (2080 bp, EcorI/PstI) + Promoter BBa_J23110/RBS_BBa_B0034 (68 bp, EcoRI/SpeI)+ Fluorene Synthetic F2: flnE, flnD1, ORF16, flnC_Ter_BBa_B0015: (3433 bp, XbaI/PstI)	>300	2
Vector alone		
Vector_pSB1C3_ (2080 bp, EcorI/PstI)	3	

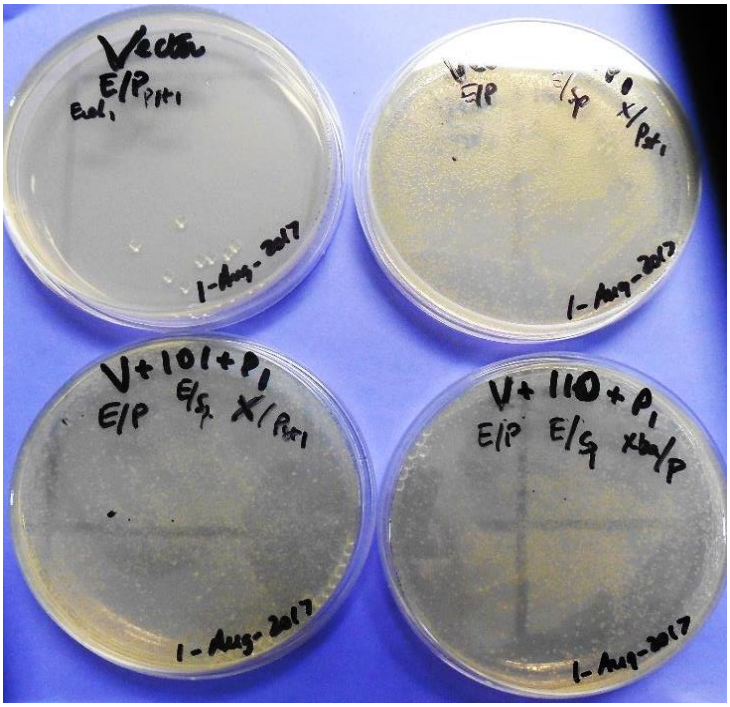


Figure. LB-Chloramphenicol plates with transformation of E.coli DH5a cells with ligation of vector pSB1C3 alone, vector pSB1C3 + promoter 100 or 101 or 110 + Phenanthrene-1 fragment.

Promoter area 100:part BBa_J23100 +RBS_BBa_B0034
Promoter area 101:part BBa_J23101 +RBS_BBa_B0034
Promoter area 110:part BBa_J23110 +RBS_BBa_B0034

Phenanthrene-1= P1: Synthetic phnF, phnE, phnC, phnD

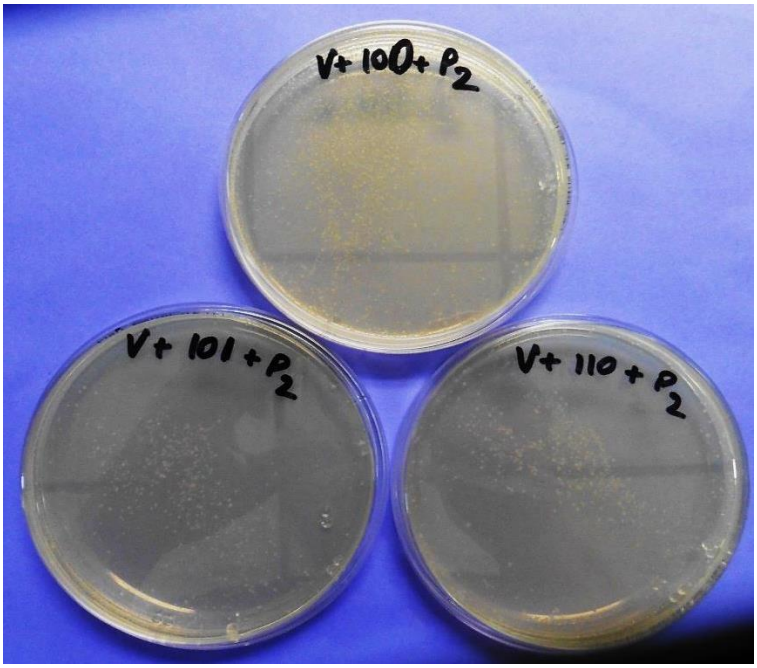


Figure. LB-Chloramphenicol plates with transformation of E.coli DH5a cells with ligation of vector pSB1C3 alone, vector >300+ promoter 100 or 101 or 110 + Phenanthrene-2 fragment.

Promoter area 100: part BBa_J23100 +RBS_BBa_B0034
Promoter area 101:part BBa_J23101 +RBS_BBa_B0034
Promoter area 110:part BBa_J23110 +RBS_BBa_B0034

Phenanthrene-2= P2: Synthetic phnAc, phnAd, phnB

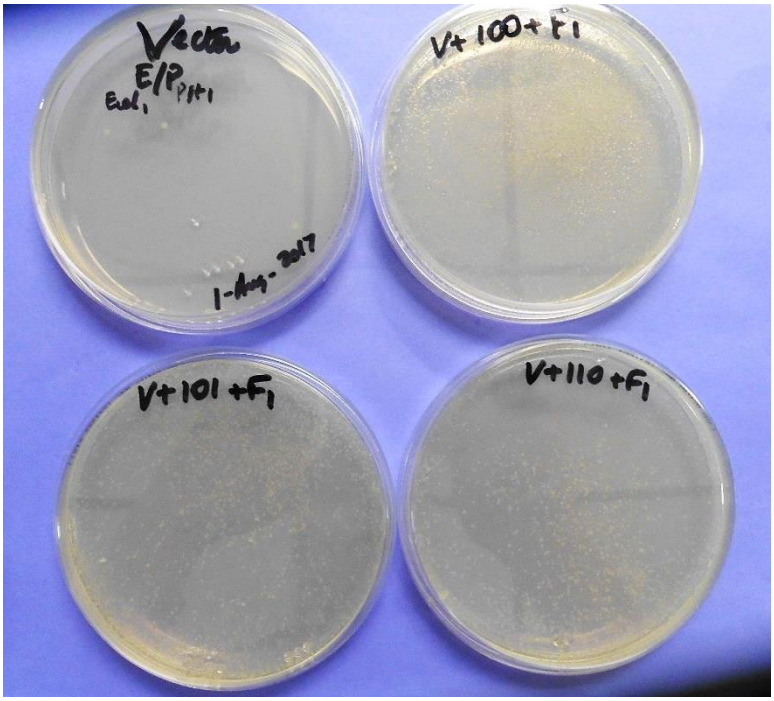


Figure. LB-Chloramphenicol plates with transformation of E.coli DH5a cells with ligation of vector pSB1C3 alone, vector + promoter pSB1C3 100 or 101 or 110 + Fluorene-1 fragment.

Promoter area 100: part BBa_J23100 +RBS_BBa_B0034
Promoter area 101:part BBa_J23101 +RBS_BBa_B0034
Promoter area 110:part BBa_J23110 +RBS_BBa_B0034

Fluorene-1= F1: Synthetic flnB, dbfA1, dbfA2

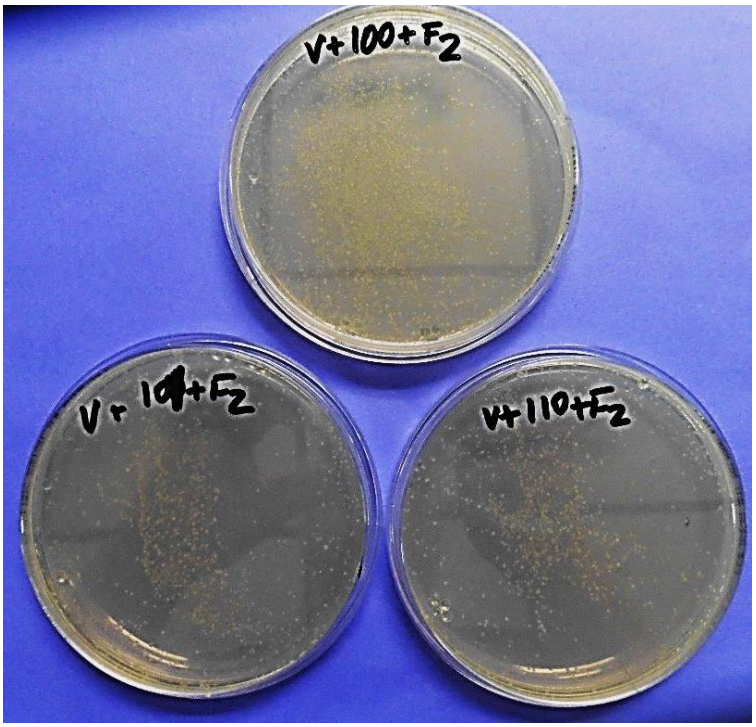


Figure. LB-Chloramphenicol plates with transformation of E.coli DH5a cells with ligation of vector pSB1C3 alone, vector pSB1C3 + promoter 100 or 101 or 110 + Fluorene-2 fragment.

Promoter area 100: part BBa_J23100 +RBS_BBa_B0034
Promoter area 101:part BBa_J23101 +RBS_BBa_B0034
Promoter area 110:part BBa_J23110 +RBS_BBa_B0034

Fluorene-2= F2: Synthetic flnE, flnD1, ORF16, flnC

5.4. Clone Verification

Date: 4-Aug-2017

Materials

a. DNA extraction kit, Zymo Research
b. LB liquid medium
c. Antibiotic stock solution (chloramphenicol 34 mg/mL)
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

Culture Set-up

a. Grow selected number of colonies in 3 mL LB medium supplemented with Chloramphenicol (35µg/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.
e. Check clones by digestion.

Set-up Digestion for Clone Verification

Date: 5-Aug-2017

a. Set up digestion as shown below.
b. This is a 1% gel agarose gel, TAE, prepared with Sybr green dye.
c. Double digestion of miniprep DNA with EcoRI/SpeI for F1+promoter area and P1+promoter area clones
d. Double digestion of miniprep DNA with XbaI/PstI for F2+promoter area and P2+promoter area clones
e. Set-up restriction digestion (20µl reaction) in a 1.5 mL tube
f. Turn on water bath at 37°C
g. Add the reagents in the order and with volume described in the table below.
h. Spin the tube briefly for 15 seconds at 10,000 rpm
i. Incubate the tube at 37°C for 15 min
j. The reaction already have a loading buffer
k. Load the digestion reaction on a 1% Agarose gel, TAE
l. After running the electrophoresis for 2 hours at 80V, cut out with a razor blade the linearized band of the desired size.
m. Purify the DNA from the gel using the Zymoclean Gel DNA Recovery Kit.
n. Elute the DNA from the Zymoclean column with 12 µl of sterile filtered H2O

Component	Volume	**Final condition**
Reagent grade water	10.5 µl	
10X BufferFastDigest Green Buffer	1.5 µl	1 X
Plasmid miniprep	1.5µl	~0.2-0.4 µg
Restriction Enzyme 1	1.0 µl	
Restriction Enzyme 2	1.0 µl	

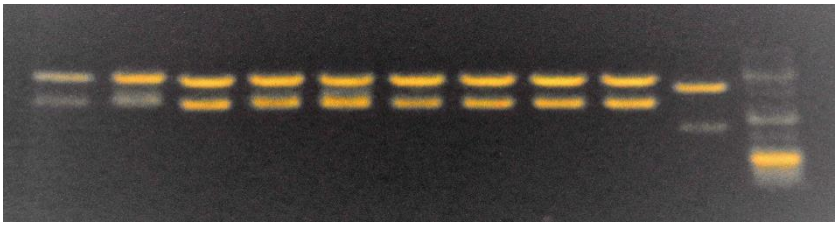
Expected Fragments Size for Verification

Phenanthrene:
EcoR/SpeI digestion for P1 based clones
XbaI/PstI digestion for P2 based clones

Description	**Expected size**
Vector (2080 bp, EcorI/PstI)) + Promoter BBa_J23100_BBa_B0034 (68 bp, EcoRI/SpeI) +	Vector + 4183 bp

Phenanthrene Synthetic P1: phnF, phnE, phnC, phnD: (4115 bp, XbaI/PstI)	
Vector (2080 bp, EcorI/PstI)) + Promoter BBa_J23101_BBa_B0034 (68 bp, EcoRI/SpeI) + Phenanthrene Synthetic P1: phnF, phnE, phnC, phnD: (4115 bp, XbaI/PstI)	Vector + 4183 bp
Vector (2080 bp, EcorI/PstI)) + Promoter BBa_J23110_BBa_B0034 (68 bp, EcoRI/SpeI) + Phenanthrene Synthetic P1: phnF, phnE, phnC, phnD: (4115 bp, XbaI/PstI)	Vector + 4183 bp
Vector (2080 bp, EcorI/PstI)) + Promoter BBa_J23100_BBa_B0034 (68 bp, EcoRI/SpeI)+ Phenanthrene Synthetic P2: phnAc, phnAd, phnB: (3062 bp, XbaI/PstI)	Vector + 4130 bp
Vector (2080 bp, EcorI/PstI)) + Promoter BBa_J23101_BBa_B0034 (68 bp, EcoRI/SpeI) +Phenanthrene Synthetic P2: phnAc, phnAd, phnB: (3062 bp, XbaI/PstI)	Vector + 4130 bp
Vector (2080 bp, EcorI/PstI)) + Promoter BBa_J23110_BBa_B0034 (68 bp, EcoRI/SpeI)+ Phenanthrene Synthetic P2: phnAc, phnAd, phnB: (3062 bp, XbaI/PstI)	Vector + 4130 bp

Gel Electrophoresis Picture for P1 derived Clones



1)Clone CCA-23 [Phenanthrene insert 1 XbaI/PstI + Promoter BBa_J23100 EcoRI/SpeI+ pSB1C3 EcoRI/PstI]
2)Clone CCA-24 [Phenanthrene insert 1 XbaI/PstI + Promoter BBa_J23100 EcoRI/SpeI+ pSB1C3 EcoRI/PstI]
3)Clone CCA-25 [Phenanthrene insert 1 XbaI/PstI + Promoter BBa_J23100 EcoRI/SpeI+ pSB1C3 EcoRI/PstI]

4)Clone CCA-26 [Phenanthrene insert 1 XbaI/PstI + Promoter BBa_J23101 EcoRI/SpeI+ pSB1C3 EcoRI/PstI]
5)Clone CCA-27[Phenanthrene insert 1 XbaI/PstI + Promoter BBa_J23101 EcoRI/SpeI+ pSB1C3 EcoRI/PstI]
6)Clone CCA-28 [Phenanthrene insert 1 XbaI/PstI + Promoter BBa_J23101 EcoRI/SpeI+ pSB1C3 EcoRI/PstI]

7)Clone CCA-29 [Phenanthrene insert 1 XbaI/PstI + Promoter BBa_J23110 EcoRI/SpeI+ pSB1C3 EcoRI/PstI]
8)Clone CCA-30 [Phenanthrene insert 1 XbaI/PstI + Promoter BBa_J23110 EcoRI/SpeI+ pSB1C3 EcoRI/PstI]
9)Clone CCA-31 [Phenanthrene insert 1 XbaI/PstI + Promoter BBa_J23110 EcoRI/SpeI+ pSB1C3 EcoRI/PstI]

10)BBa_J04450, pSB3T5 Tetracycline, reporter gene, EcoRI- PstI
11) DNA Ladder

Result for P1 cloning:

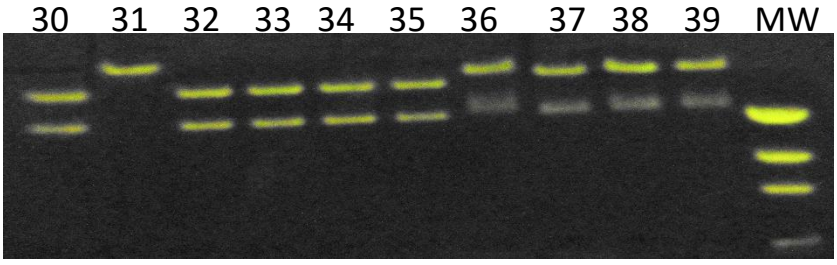
CCA-23 , CCA-26, and CCA29 were first checked for presence of insert using a EcoRI/PstI digestion. As an insert of the correct size was observed on the gel, they were selected for the second step of cloning and digested with SpeI/PstI.

Gel Electrophoresis Picture for F1 and F2 and P2 derived Clones

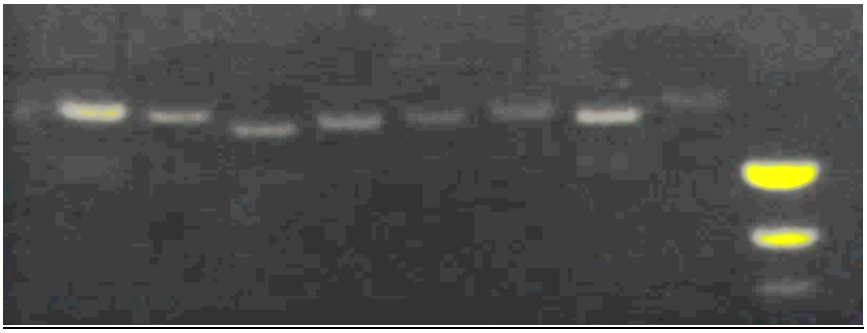
Electrophoresis Gel 1

Upper Gel 1
1) DNA Miniprep Clone CCA-30 Digestion EcoRI/SpeI [Fluorene insert 1 + Promoter BBa_J23100 + pSB1C3]
2) DNA Miniprep Clone CCA-31 Digestion EcoRI/SpeI [Fluorene insert 1 + Promoter BBa_J23100 + pSB1C3]
3) DNA Miniprep Clone CCA-32 Digestion EcoRI/SpeI [Fluorene insert 1 + Promoter BBa_J23101 + pSB1C3]
4) DNA Miniprep Clone CCA-33 Digestion EcoRI/SpeI [Fluorene insert 1 + Promoter BBa_J23101 + pSB1C3]
5) DNA Miniprep Clone CCA-34 Digestion EcoRI/SpeI [Fluorene insert 1 + Promoter BBa_J23110 + pSB1C3]
6) DNA Miniprep Clone CCA-35 Digestion EcoRI/SpeI [Fluorene insert 1 + Promoter BBa_J23110 + pSB1C3]
7) DNA Miniprep Clone CCA-36 Digestion XbaI/PstI [Fluorene insert 2 + Promoter BBa_J23100 + pSB1C3]
8) DNA Miniprep Clone CCA-37 Digestion XbaI/PstI [Fluorene insert 2 + Promoter BBa_J23100 + pSB1C3]
9) DNA Miniprep Clone CCA-38 Digestion XbaI/PstI [Fluorene insert 2 + Promoter BBa_J23101 + pSB1C3]
10) DNA Miniprep Clone CCA-39 Digestion XbaI/PstI [Fluorene insert 2 + Promoter BBa_J23101 + pSB1C3]
11) DNA Ladder (Molecular Weight Marker)

Lower Gel 1
1) DNA Miniprep Clone CCA-40 Digestion EcoRI/SpeI [Fluorene insert 2 + Promoter BBa_J23110 + pSB1C3]
2) DNA Miniprep Clone CCA-41 Digestion XbaI/PstI [Fluorene insert 2 + Promoter BBa_J23110 + pSB1C3]
3) DNA Miniprep Clone CCA-42 Digestion XbaI/PstI [Phenanthrene insert 2 + Promoter BBa_J23100 + pSB1C3]
4) DNA Miniprep Clone CCA-43 Digestion XbaI/PstI [Phenanthrene insert 2 + Promoter BBa_J23100 + pSB1C3]
5) DNA Miniprep Clone CCA-44 Digestion XbaI/PstI [Phenanthrene insert 2 + Promoter BBa_J23101 + pSB1C3]
6) DNA Miniprep Clone CCA-45 Digestion XbaI/PstI [Phenanthrene insert 2 + Promoter BBa_J23101 + pSB1C3]
7) DNA Miniprep Clone CCA-46 Digestion XbaI/PstI [Phenanthrene insert 2 + Promoter BBa_J23110 + pSB1C3]
8) DNA Miniprep Clone CCA-47 Digestion XbaI/PstI [Phenanthrene insert 2 + Promoter BBa_J23110 + pSB1C3]
9) DNA Ladder (Molecular Weight Marker)



40 41 42 43 44 45 46 47 48 49 MW



5.4. CLONES DESCRIPTION

The clones that were kept are the following:

Clone CCA-23 [Phenanthrene insert 1 XbaI/PstI + Promoter BBa_J23100 EcoRI/SpeI+ pSB1C3 EcoRI/PstI]
Clone CCA-26 [Phenanthrene insert 1 XbaI/PstI + Promoter BBa_J23101 EcoRI/SpeI+ pSB1C3 EcoRI/PstI]
Clone CCA-29 [Phenanthrene insert 1 XbaI/PstI + Promoter BBa_J23110 EcoRI/SpeI+ pSB1C3 EcoRI/PstI]

Clone CCA-42 [Phenanthrene insert 2 XbaI/PstI + Promoter BBa_J23100 EcoRI/SpeI+ pSB1C3 EcoRI/PstI]
Clone CCA-44 [Phenanthrene insert 2 XbaI/PstI + Promoter BBa_J23101 EcoRI/SpeI+ pSB1C3 EcoRI/PstI]
Clone CCA-46 [Phenanthrene insert 2 XbaI/PstI + Promoter BBa_J23110 EcoRI/SpeI+ pSB1C3 EcoRI/PstI]

Clone CCA-30 [Fluorene insert 1 XbaI/PstI + Promoter BBa_J23100 EcoRI/SpeI+ pSB1C3 EcoRI/PstI]
Clone CCA-32 [Fluorene insert 1 XbaI/PstI + Promoter BBa_J23101 EcoRI/SpeI+ pSB1C3 EcoRI/PstI]
Clone CCA-34 [Fluorene insert 1 XbaI/PstI + Promoter BBa_J23110 EcoRI/SpeI+ pSB1C3 EcoRI/PstI]

Clone CCA-36 [Fluorene insert 2 XbaI/PstI + Promoter BBa_J23100 EcoRI/SpeI+ pSB1C3 EcoRI/PstI]
Clone CCA-38 [Fluorene insert 2 XbaI/PstI + Promoter BBa_J23101 EcoRI/SpeI+ pSB1C3 EcoRI/PstI]
Clone CCA-40 [Fluorene insert 2 XbaI/PstI + Promoter BBa_J23110 EcoRI/SpeI+ pSB1C3 EcoRI/PstI]

6- CLONING OF FULL LENGTH (SAME PLASMID) POLYCISTRONIC F1, F2 (FLUORENE) AND P1, P2 (PHENANTHRENE) BEHIND CONSTITUTIVE PROMOTERS

6.1. PHENANTHRENE

6.1.1. Cloning of Phenanthrene Full Length (P1 and P2 on same plasmid)

6.1.1.1. Preparation of fragments for Phenanthrene Cloning

Source of clones:

The 2 clones CCA-23 and CCA-42 obtained as shown above were used to prepare a plasmid DNA containing the full length phenanthrene pathway under the control of promoter BBa_J23100 Miniprep DNA of CCC-23 was digested with SpeI/PstI and miniprep DNA of CCC-42 was digested with Xba/PstI to release BBa-J23100_RBS_P2.

Clone CCA-23 [Phenanthrene insert 1 XbaI/PstI + Promoter BBa_J23100 EcoRI/SpeI+ pSB1C3 EcoRI/PstI]
Clone CCA-42 [Phenanthrene insert 2 XbaI/PstI + Promoter BBa_J23100 EcoRI/SpeI+ pSB1C3 EcoRI/PstI]

The 2 clones CCA-26 and CCA-44 obtained as shown above were used to prepare a plasmid DNA containing the full length phenanthrene pathway under the control of promoter BBa_J23101
Miniprep DNA of CCC-26 was digested with SpeI/PstI and miniprep DNA of CCC-44 was digested with Xba/PstI to release BBa-J23101_RBS_P2.
Clone CCA-26 [Phenanthrene insert 1 XbaI/PstI + Promoter BBa_J23101 EcoRI/SpeI+ pSB1C3 EcoRI/PstI]
Clone CCA-44 [Phenanthrene insert 2 XbaI/PstI + Promoter BBa_J23101 EcoRI/SpeI+ pSB1C3 EcoRI/PstI]

The 2 clones CCA-29 and CCA-46 obtained as shown above were used to prepare a plasmid DNA containing the full length phenanthrene pathway under the control of promoter BBa_J23110.
Miniprep DNA of CCC-29 was digested with SpeI/PstI and miniprep DNA of CCC-46 was digested with Xba/PstI to release BBa-J23110_RBS_P2.
Clone CCA-29 [Phenanthrene insert 1 XbaI/PstI + Promoter BBa_J23110 EcoRI/SpeI+ pSB1C3 EcoRI/PstI]
Clone CCA-46 [Phenanthrene insert 2 XbaI/PstI + Promoter BBa_J23110 EcoRI/SpeI+ pSB1C3 EcoRI/PstI]

a. The clones listed above were the sources of DNA for these steps of cloning.
b. Double Digestion of recipient vector already containing P1 with SpeI and PstI
c. Double Digestion of clones containing P2 with XbaI/PstI
d. Restriction Digest Set-up (20µl reaction) in a 1.5 mL tube
e. Add the reagents as described below.
f. Incubate the tube at 37°C for 15 min
g. Note: there is no need to add a loading buffer because the digestion buffer already has it.
h. Load the reaction on a 1% Agarose gel, TAE
i. After running the electrophoresis for 2 hours at 80V, cut the linearized band.
j. Purify the DNA from the gel using the Zymoclean Gel DNA Recovery Kit
k. Elute the DNA from the Zymoclean column with 12 µl of sterile filtered H2O

P1 digestion condition:

Component	Volume	**Final condition**
Reagent grade water	9.5 µl	
10X Buffer	1.5 µl	1x
P1 Clones (CCA-23, CCA-26 and CCA-29)	2.0 µl	~ 0.3 µg
SpeI	1.0 µl	
PstI	1.0 µl	

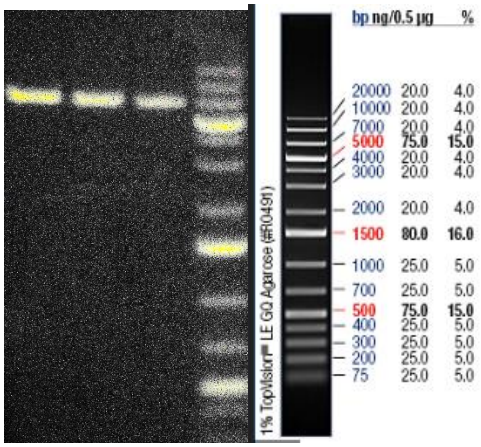
P2 digestion condition:

Component	Volume	**Final condition**
Reagent grade water	9.5 µl	
10X Buffer	1.5 µl	1x
P2 Clones (CCA-42, CCA-44 and CCA-46)	2.0 µl	~ 0.3 µg
XbaI	1.0 µl	
PstI	1.0 µl	

Electrophoresis Gel 1

Preparation of recipient plasmid:
1) DNA Miniprep Clone CCA-23 Digestion SpeI/PstI [Phenanthrene insert 1 + Promoter are BBa_J23100_RBS + pSB1C3]
2) DNA Miniprep Clone CCA-26 Digestion SpeI/PstI [Phenanthrene insert 1 + Promoter BBa_J23101_RBS + pSB1C3]
3) DNA Miniprep Clone CCA-29 Digestion SpeI/PstI [Phenanthrene insert 1 + Promoter BBa_J23110_RBS + pSB1C3]
4) DNA Ladder (Molecular Weight Marker)
5) No gel loading

1 2 3 MW



6.1.1.2. Ligation Phenanthrene Cloning

Date: 1-Aug-2017 and 2-Aug-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	6.0 µl
10X T4 ligation buffer	1.0 µl
SpeI/PstI Linearized recipient vector- pSB1C3-P1 under the control of promoters and RBS BBa_J23100 BBa_J23101 BBa_J23110	0.5 µl
XbaI/PstI - fragment pSB1C3-P2 under the control of promoters and RBS: BBa_J23100 BBa_J23101 BBa_J23110	2.0 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
SpeI/PstI Linearized recipient vector- pSB1C3-P1 under the control of promoters and RBS BBa_J23100	0.5 µl

BBa_J23101 BBa_J23110	
T4 DNA Ligase (5Weiss/µl)	0.5 µl

6.1.2. Transformation

Date: 2-Aug-2017 and 3-Aug-2017

Materials

a. LB Chloramphenicol 34 µg/mL agar plates, Cat No. Teknova, L1017
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
g. Ice bucket and ice
h. Water bath (42°C)
i. Incubator (37°C)

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 5 µl ligation mix to 50 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 SOC medium 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 Chloramphenicol 34 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.

6.1.3. Transformation Results

Date: 2-Aug-2017 and 3-Aug-2017

Description [ligation]	**Number of colonies (50 µL volume plating)**	**Number of clones analyzed by digestion**	**Clones Designation**
Control: Recipient vector containing P1_BBa_J23100_RBS_Synthetic phnF, phnE, phnC, phnD_ pSB1C3 [SpeI/PstI]	0		
Ligation: Recipient vector containing P1_BBa_J23100_RBS_Synthetic phnF, phnE, phnC, phnD_ pSB1C3 [SpeI/PstI] + Synthetic phnAc, phnAd, phnB [XbaI/PstI]	>100	3	CCA-57 CCA-58 CCA-59
Control: Recipient vector containing P1_BBa_J23101_RBS_Synthetic phnF, phnE, phnC, phnD_ pSB1C3 [SpeI/PstI]	0		
Ligation: Recipient vector containing P1_BBa_J23101_RBS_Synthetic phnF, phnE, phnC, phnD_ pSB1C3 [SpeI/PstI] + Synthetic phnAc, phnAd, phnB [XbaI/PstI]	>100	3	CCA-60 CCA-61 CCA-62
Control: Recipient vector containing P1_BBa_J23110_RBS_Synthetic phnF, phnE, phnC, phnD_ pSB1C3 [SpeI/PstI]	0		
Ligation: Recipient vector containing P1_BBa_J23110_RBS_Synthetic phnF, phnE, phnC, phnD_ pSB1C3 [SpeI/PstI] + Synthetic phnAc, phnAd, phnB [XbaI/PstI]	>100	3	CCA-63 CCA-64 CCA-65

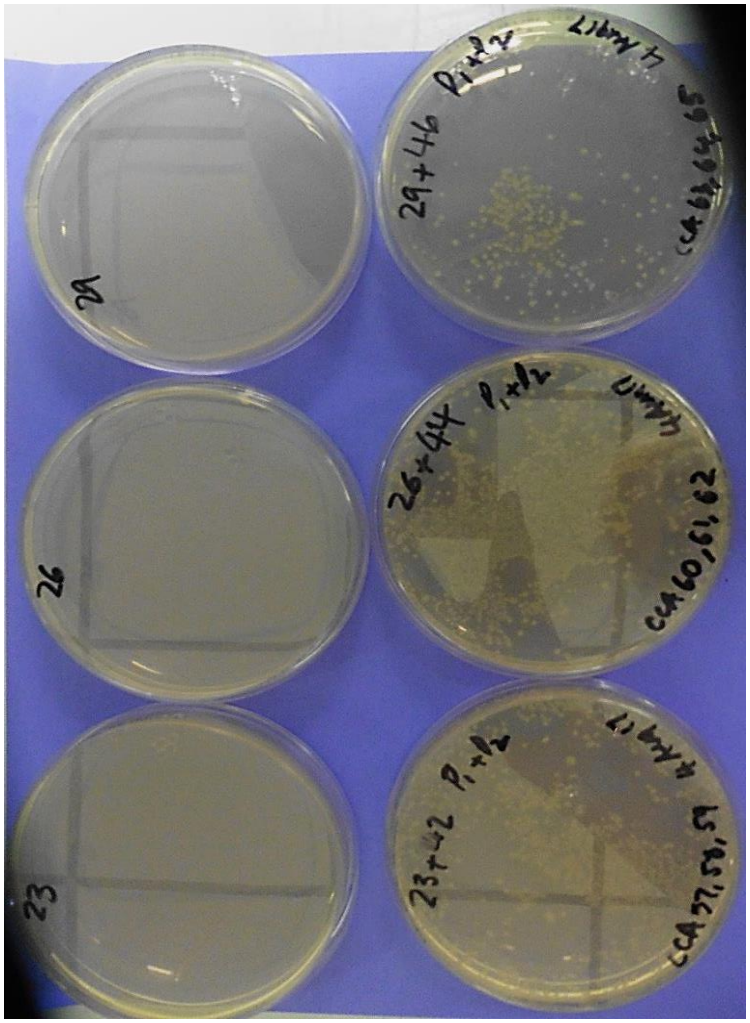


Figure. LB-Chloramphenicol plates with E.coli DH5a cells transformed with product of ligation of pSBB1C3 containing Phenanthrene-1 (SpeI/PstI) ligated to Phenanthrene-2 (XbaI/PstI) under the control of the constitutive promoter BBa_J23100 [100] or BBa_J23101 [101] or BBa_J23110[110] and RBS.

Phenanthrene-1= P1: Synthetic phnF, phnE, phnC, phnD
Phenanthrene-2= P2: Synthetic phnAc, phnAd, phnB

6.1.4. Clone Verification

Date: 5-Aug-2017/6-Aug-2017

Clone verification is done by digestion. Cultures are set up to extract DNA.

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 chloramphenicol (34 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.

Clones

Analysis of Individual clones: CCA_57; CCA_58; CCA_59
BBa_J23100_RBS_P1_Terminator + BBa_J23100_RBS_P2_Terminator in vector pSB1C3

Analysis of Individual clones: CCA_60; CCA_61; CCA_62
BBa_J23101_RBS_P1_Terminator + BBa_J23101_RBS_P2_Terminator in vector pSB1C3

Analysis of Individual clones: CCA_63; CCA_64; CCA_65
BBa_J23110_RBS_P1_Terminator + BBa_J23110_RBS_P2_Terminator in vector pSB1C3

P1: Synthetic phnF, phnE, phnC, phnD_Terminator
P2: Synthetic phnAc, phnAd, phnB-Terminator

Set-up Digestion for Clone Verification

Date: 5-Aug-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 EcoRI/PstI
d. Set-up restriction digestion (15µl 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.

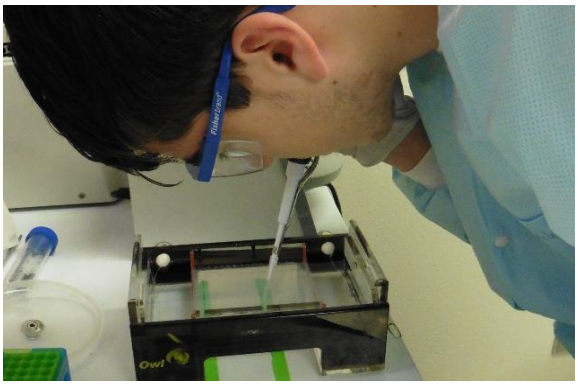
Component	Volume	**Final condition**
Reagent grade water	9.5 µl	
10X BufferFastDigest Green Buffer	1.5 µl	1 X
Plasmid miniprep	1.5 µl	~0.2-0.4 µg
Restriction Enzyme 1	1.0 µl	
Restriction Enzyme 2	1.0 µl	

Expected Fragments Size for Verification

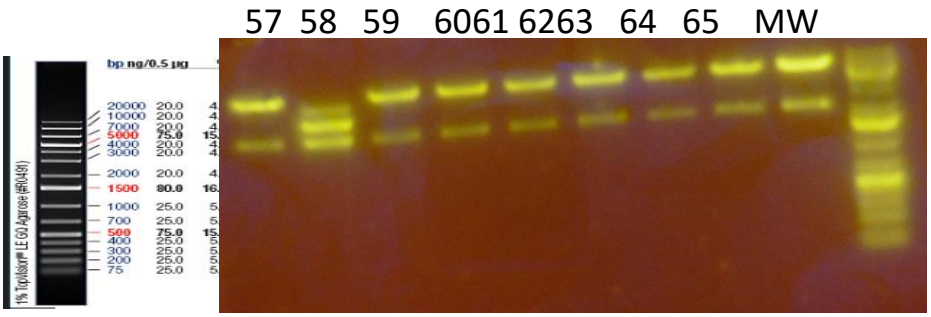
Phenanthrene clones:
EcoRI/PstI digestion: 2 fragments should be observed: the vector pSB1C3 and P1+P2 (4165 bp+3057bp)

P1_Terminator: XbaI/Pst1: 4115 bp
Promoter_RBS_P1_Terminator: 4183 bp
Promoter_RBS_P1_Terminator: 4165 bp EcoRI/SpeI

P2_Terminator: XbaI/Pst1: 3022 bp
Promoter_RBS_P2_Terminator: 3090 bp
Promoter_RBS_P2_Terminator: 3057 bp EcoRI/SpeI



Results


57 58 59 6061 6263 64 65 MW
bp ng/0.5 µg
20000 20.0
10000 20.0
7000 20.0
5000 75.0
4000 20.0
3000 20.0
2000 20.0
1500 80.0
1000 25.0
700 25.0
500 75.0
400 25.0
300 25.0
200 25.0
75 25.0

6.2. FLUORENE

6.2.1. Cloning of Fluorene Full Length (F1 and F2) into a Vector with p15a Ori

6.2.1.1. Preparation of fragments for Fluorene Cloning

Source of clones:

The 2 clones CCA-30 and CCA-36 obtained as shown above were used to prepare a plasmid DNA containing the full length fluorene pathway under the control of promoter BBa_J23100.
Miniprep DNA of CCC-30 was digested with EcoRI/SpeI to release BBa-J23100_RBS_F1 and miniprep DNA of CCC-36 and was digested with Xba/PstI to release BBa-J23100_RBS_F2.
Clone CCA-30 [Fluorene insert 1 XbaI/PstI + Promoter BBa_J23100 EcoRI/SpeI+ pSB1C3 EcoRI/PstI]
Clone CCA-36 [Fluorene insert 2 XbaI/PstI + Promoter BBa_J23100 EcoRI/SpeI+ pSB1C3 EcoRI/PstI]

The 2 clones CCA-32 and CCA-38 obtained as shown above were used to prepare a plasmid DNA containing the full length fluorene pathway under the control of promoter BBa_J23101.
Miniprep DNA of CCC-32 was digested with EcoRI/SpeI to release BBa-J23101_RBS_F1 and miniprep DNA of CCC-38 and was digested with Xba/PstI to release BBa-J23101_RBS_F2.
Clone CCA-32 [Fluorene insert 1 XbaI/PstI + Promoter BBa_J23101 EcoRI/SpeI+ pSB1C3 EcoRI/PstI]
Clone CCA-38 [Fluorene insert 2 XbaI/PstI + Promoter BBa_J23101 EcoRI/SpeI+ pSB1C3 EcoRI/PstI]

The 2 clones CCA-34 and CCA-40 obtained as shown above were used to prepare a plasmid DNA containing the full length fluorene pathway under the control of promoter BBa_J23110.
Miniprep DNA of CCC-34 was digested with EcoRI/SpeI to release BBa-J23110_RBS_F1 and miniprep DNA of CCC-38 and was digested with Xba/PstI to release BBa-J23110_RBS_F2.
Clone CCA-34 [Fluorene insert 1 XbaI/PstI + Promoter BBa_J23110 EcoRI/SpeI+ pSB1C3 EcoRI/PstI]
Clone CCA-40 [Fluorene insert 2 XbaI/PstI + Promoter BBa_J23110 EcoRI/SpeI+ pSB1C3 EcoRI/PstI]

Plasmid pSB3T5 (available with part BBa_J04450 in 2017 distribution plate) which contain p15a as origin of replication was digested with EcoRI and PstI.

Methods:

a. Double Digestion of F1 under the control of one of the 3 promoters with EcoRI/SpeI
b. Double Digestion of F2 under the control of one of the 3 promoters with XbaI/PstI
c. Double Digestion of recipient vector with EcorRI/PstI
d. Restriction Digest Set-up (20µl reaction) in a 1.5 mL tube
e. Add the reagents as described below.
f. Incubate the tube at 37°C for 15 min
g. Note: there is no need to add a loading buffer because the digestion buffer already has it.
h. Load the reaction on a 1% Agarose gel
i. After running the electrophoresis for 2 hours at 80V, cut the linearized band.
j. Purify the DNA from the gel using the Zymoclean Gel DNA Recovery Kit
k. Elute the DNA from the Zymoclean column with 12 µl of sterile filtered H2O

F1 digestion condition:

Component	Volume	**Final condition**

Reagent grade water	9.5 µl	
10X Buffer	1.5 µl	1x
F1 Clones (CCA-30, CCA-32 and CCA-34)	2.0 µl	~ 0.3 µg
EcoRI	1.0 µl	
SpeI	1.0 µl	

F2 digestion condition:

Component	Volume	**Final condition**
Reagent grade water	9.5 µl	
10X Buffer	1.5 µl	1x
F1 Clones (CCA-36, CCA-38 and CCA-40)	2.0 µl	~ 0.3 µg
XbaI	1.0 µl	
PstI	1.0 µl	

Vector digestion condition:

Component	Volume	**Final condition**
Reagent grade water	9.5 µl	
10X Buffer	1.5 µl	1x
Vector	2.0 µl	~ 0.3 µg
EcoRI	1.0 µl	
PstI	1.0 µl	

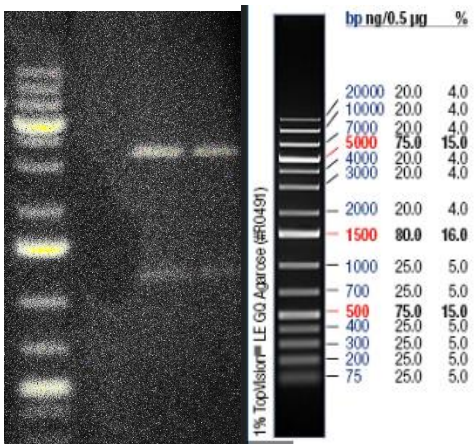
Electrophoresis Gel 1

4) DNA Ladder (Molecular Weight Marker)
5) No gel loading

Preparation of vector in duplicate:
6) pSB3T5 (p15 a) vector EcoRI/PstI

7) pSB3T5 (p15 a) vector EcoRI/PstI

MW567



6.2.1.2. Ligation Fluorene Cloning

Date: 1-Aug-2017 and 2-Aug-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
Linearized Recipient Vector EcoRI/PstI (Tetracycline)	0.5 µl
EcoRI/SpeI - F1 insert under the control of promoters and RBS	1.5 µl

BBa_J23100 BBa_J23101 BBa_J23110	
XbaI/PstI – F2 insert under the control of promoters and RBS: BBa_J23100 BBa_J23101 BBa_J23110	1.5µl
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
Linearized Recipient Vector EcoRI/PstI (Tetracycline)	0.5 µl
T4 DNA Ligase (5Weiss/µl)	0.5µl

6.2.1.3. Transformation

Date: 4-Aug-2017

Materials

a. LB Tetracycline 12.5 µg/mL agar plates, Cat No. Teknova, L5072
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
g. Ice bucket and ice
h. Water bath (42°C)
i. Incubator (37°C)

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 5 µl ligation mix to 50 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 SOC medium 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 tetracycline12.5 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

6.2.1.4. Transformation Results

Date: 5-Aug-2017

Description [ligation]	**Number of colonies (50 µL volume plating)**	**Number of clones analyzed by digestion**	**Clones Designation**
Control: Recipient vector [EcoRI/PstI]	0		
Ligation: Recipient vector p15a + F1 +F2 each under control of promoter BBa_J23100	>100	3	CCA-48 CCA-49 CCA-50
Control: Recipient vector [EcoRI/PstI]	0		
Ligation: Recipient vector p15a + F1 +F2 each under control of promoter BBa_J23101	>100	3	CCA-51 CCA-52 CCA-53
Control: Recipient vector [EcoRI/PstI]	0		
Ligation: Recipient vector p15a + F1 +F2 each under control of promoter BBa_J23110	>100	3	CCA-54 CCA-55 CCA-56

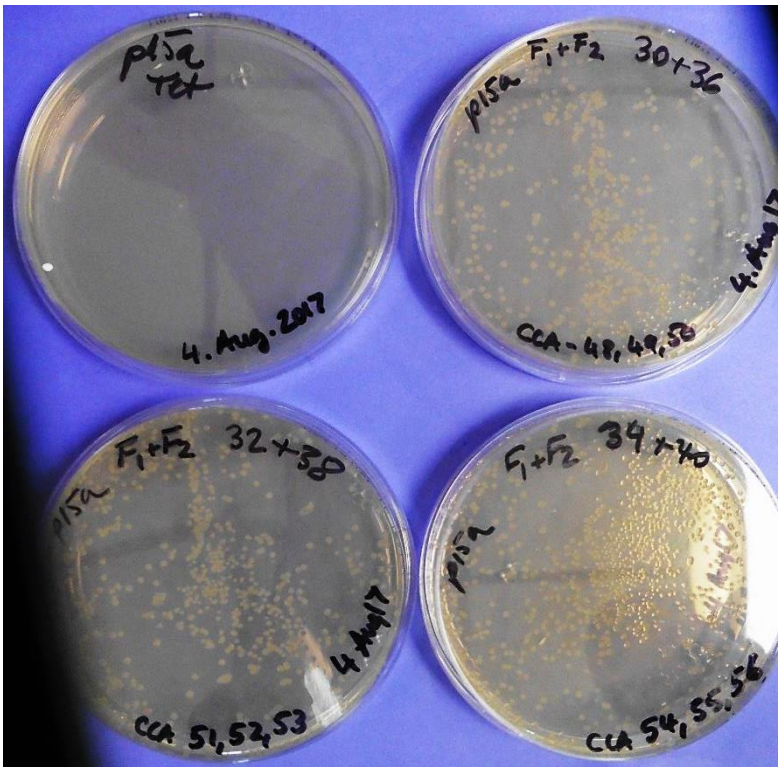


Figure. LB-Tetracycline plates with E.coli DH5a cells transformed with product of ligation of p15aTet (vector pSB3T5) (EcoRI/PstI) ligated to Fluorene-1 (EcoRI/SpeI) and ligated to Fluroene-2 (XbaI/PstI) under the control of the constitutive promoter BBa_J23100 [100] or BBa_J23101 [101] or BBa_J23110[110].

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

Fluorene-1= Promoter_RBS_Synthetic flnB, dbfA1, dbfA2
Fluorene-2= Promoter_RBS_Synthetic flnE, flnD1, ORF16, flnC

6.2.1.5. Clone Verification

Date: 6-Aug-2017/7-Aug-2017

Clone verification is done by digestion. Cultures are set up to extract DNA.

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 tetracycline (12.5µg/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.

Clones
Analysis of Individual clones: CCA_48; CCA_49; CCA_50
BBa_J23100_RBS_F1_Terminator + BBa_J23100_RBS_F2_Terminator in vector pSB3T5

Analysis of Individual clones: CCA_51; CCA_52; CCA_53
BBa_J23101_RBS_F1_Terminator + BBa_J23101_RBS_F2_Terminator in vector pSB3T5

Analysis of Individual clones: CCA_54; CCA_55; CCA_56
BBa_J23110_RBS_F1_Terminator + BBa_J23110_RBS_F2_Terminator in vector pSB3T5

F1: Synthetic flnB, dbfA1, dbfA2
F2: Synthetic flnE, flnD1, ORF16, flnC

Set-up Digestion for Clone Verification
Date: 7-Aug-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/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 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	15.0 µl	
10X BufferFastDigest Green Buffer	1.5 µl	1 X
Plasmid miniprep	1.5 µl	~0.2-0.4 µg
Restriction Enzyme 1	1.0 µl	
Restriction Enzyme 2	1.0 µl	

Expected Fragments Size for Verification

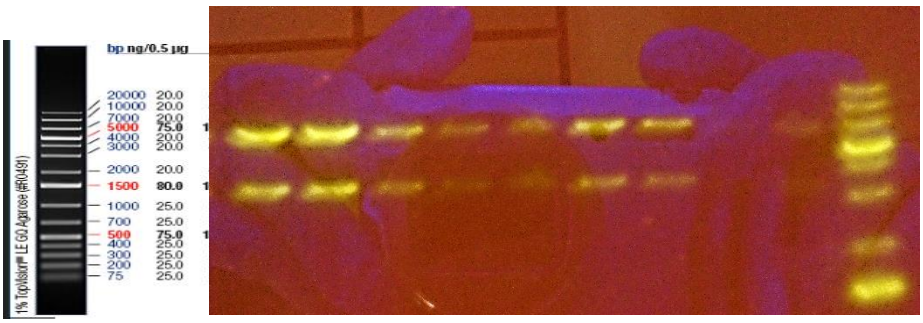
Fluorene: EcoRI/PstI digestion:
2 fragments should be observed: the vector pSB3T5 and F1+F2 (3157bp+3468 bp)

F1_Terminator: XbaI/Pst1: 3107 bp
Promoter_RBS_F1_Terminator: 3175 bp
Promoter_RBS_F1_Terminator: 3157 bp EcoRI/SpeI

F2_Terminator: XbaI/Pst1: 3433 bp
Promoter_RBS_F_Terminator2: 3501 bp
Promoter_RBS_F2_Terminator: 3468 bp EcoRI/SpeI

Results
The DNA digestion of clones CCA-48 to CCA-58 indicated that the fragments of the correct size were obtained.
Clone CCA-48, CCA51, and CCA54 will be sent out for sequencing for verification.

CCA-48 49 50 5152 5354 55 56 MW



7- SEQUENCING

Date: 8-Aug-2017
An aliquot of the DNA plasmid mini-prep was sent out for sequencing.
All files are attached below.
The sequencing data indicated that the sequence of the clones was correct.

8- TRANSFORMATION OF FULL LENGTH PHENANTHRENE AND FLUORENE CLONES INTO E.COLI BL-21

Date: 10-Aug-2017

8.1. MATERIALS

a. DNA miniprep
b. LB agar plates, Cat No. Teknova, appropriate antibiotic
c. E coli BL21 DE3, Life Technology, Cat No. 60106-1
d. SOC (Recovery Medium), Lucigen, Cat No. F98226
e. 15 mL culture tube
f. Vortex
g. Tooth pick
h. Incubator shaker
i. 42°C Water bath
j. Ice and ice bucket
k. Pipet

8.2. METHODS

a. Transform 1 µL of DNA mini-preparation into E coli BL21 DE3 chemically competent cells
b. For double transformation, transform with of 1 µL of each DNA mini-preparation into E coli BL21 DE3 chemically competent cells
c. Turn on incubator-shaker at 37°C.
d. Turn on incubator for plates at 37°C.
e. Set up water bath at 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 SOC medium and transfer mixture to 15mL Falcon tube
n. Incubate in shaker at 37°C, 225 rpm for 30 min
o. Plate 1 volumes (~100µL) of the mixture onto one LB agar plates supplemented with Chloramphenicol 35 µg/mL or Tetracycline 12.5 µg/mL or both Chloramphenicol 35 µg/mL and Tetracycline 12.5 µg/mL depending on the clone
p. Incubate plates at 37°C overnight
q. Initiate cultures in LB with the appropriate antibiotic from individual clones to analyze clones by digestion

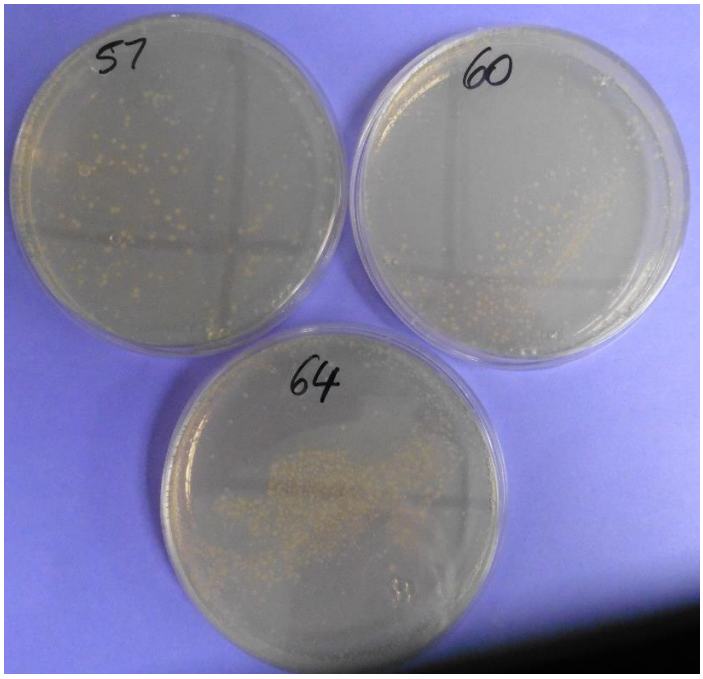


Figure. LB-Chloramphenicol plates with E.coli Bl-21 cells transformed with product mini-prep DNA of clone CCA-57, CCA 60, and CCA 64.

Clone_57: BBa_J23100_RBS_P1_Terminator + BBa_J23100_RBS_P2_Terminator in vector pSB1C3

CCA_60: BBa_J23101_RBS_P1_Terminator + BBa_J23101_RBS_P2_Terminator in vector pSB1C3

CCA_64:BBa_J23110_RBS_P1_Terminator + BBa_J23110_RBS_P2_Terminator in vector pSB1C3

Phenanthrene-1= Synthetic phnF, phnE, phnC, phnD
Phenanthrene-2= Synthetic phnAc, phnAd, phnB

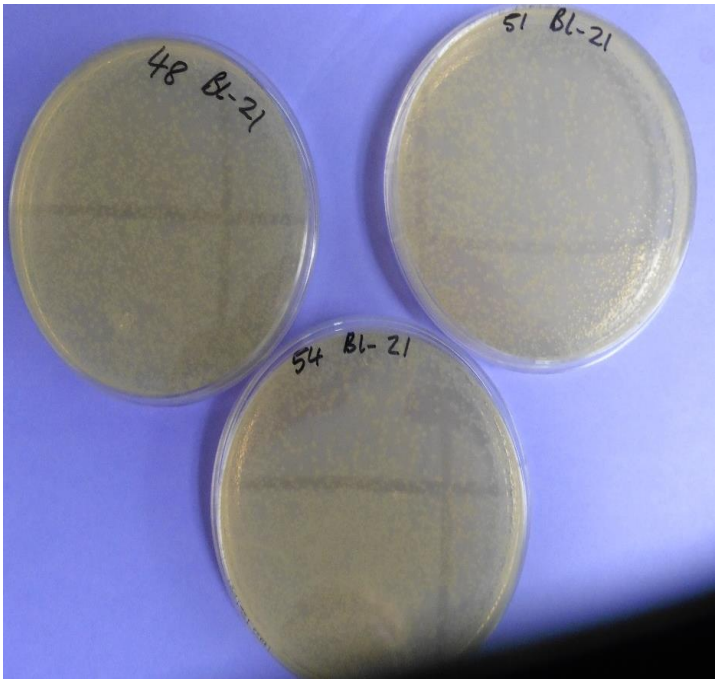


Figure. LB-Tetracycline plates with E.coli Bl-21 cells transformed with product mini-prep DNA of clone CCA-48, CCA 51, and CCA 54.

CCA_48=BBa_J23100_RBS_F1_Terminator + BBa_J23100_RBS_F2_Terminator in vector pSB3T5

CCA_51=BBa_J23101_RBS_F1_Terminator + BBa_J23101_RBS_F2_Terminator in vector pSB3T5

CCA_54=BBa_J23110_RBS_F1_Terminator + BBa_J23110_RBS_F2_Terminator in vector pSB3T5

Fluorene-1= Promoter_RBS_Synthetic flnB, dbfA1, dbfA2
Fluorene-2= Promoter_RBS_Synthetic flnE, flnD1, ORF16, flnC

9- CLONING OF FLUORENE FULL LENGTH F1 AND F2 INTO pSB1C3 FOR DEPOSIT IN REGRISTRY

9.1. PREPARATION OF DNA FRAGMENTS FOR CLONING OF FLUORENE F1 AND F2 INTO pSB1C3 FOR DEPOSIT IN REGISTRY

Source of clones:

DNA of CCA-48, CCA-51, and CCA 54 containing the full fluorene pathway in the pSB3T5 vector are the source of materials.

Methods:

a. Double Digestion of CCA-48, CCA-51, and CCA 54 under the control of one of the 3 promoters with EcoRI/PstI
b. Double Digestion of recipient vector pSB1C3 with EcorRI/PstI
c. Restriction Digest Set-up (20µl reaction) in a 1.5 mL tube
d. Add the reagents as described below.
e. Incubate the tube at 37°C for 15 min
f. Note: there is no need to add a loading buffer because the digestion buffer already has it.
g. Load the reaction on a 1% Agarose gel
h. After running the electrophoresis for 2 hours at 80V, cut the linearized band.
i. Purify the DNA from the gel using the Zymoclean Gel DNA Recovery Kit
j. Elute the DNA from the Zymoclean column with 12 µl of sterile filtered H2O

digestion condition:

Component	Volume	**Final condition**
Reagent grade water	9.5 µl	
10X Buffer	1.5 µl	1x
CCA-48, CCA-51, and CCA 54	2.0 µl	~ 0.3 µg
EcoRI	1.0 µl	
PstI	1.0 µl	

Vector digestion condition:

Component	Volume	**Final condition**
Reagent grade water	9.5 µl	
10X Buffer	1.5 µl	1x
Vector	2.0 µl	~ 0.3 µg

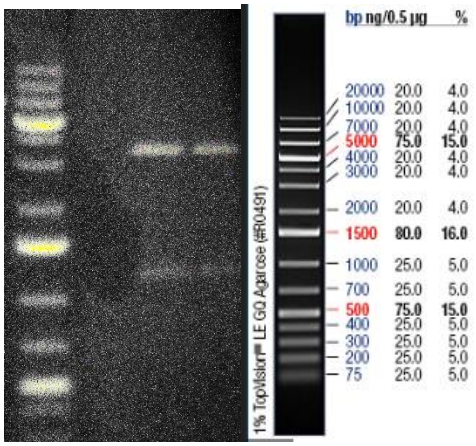
EcoRI	1.0 µl	
PstI	1.0 µl	

Electrophoresis Gel 1

4) DNA Ladder (Molecular Weight Marker)
5) No gel loading

Preparation of vector in duplicate:
6) pSB3T5 (p15 a) vector EcoRI/PstI
7) pSB3T5 (p15 a) vector EcoRI/PstI

MW567



9.2. LIGATION OF DNA FRAGMENTS F1 AND F2 INTO pSB1C3

Date: 1-Sep-2017 and 2-Sep-2017

Materials

h. T4 DNA Ligation Buffer. Invitrogen, Cat No. 46-0114
i. T4 DNA Ligase, Thermo Scientific, Cat No. K1231
j. Reagent grade water, NERL, Cat No. 98555
k. 1.5 mL tube
l. Vortex
m. Pipet and tips
n. Ice bucket and ice

Methods

d. Set ligation as shown below in 1.5 mL tube.
e. The tubes were incubated at room temperature for 1 hour.

f. The tubes were then transferred to ice.

Ligation Condition (with insert)

Component	**Volume (**µl)
Reagent grade water	5.5 µl
10X T4 ligation buffer	1.0 µl
Linearized Recipient Vector EcoRI/PstI (Chloramphenicol)	0.5 µl
Linearized insert	2.0µl
T4 DNA Ligase (5Weiss/µl)	1 µl

Control Ligation Condition (no insert)

Component	**Volume (**µl)
Reagent grade water	8.0 µl
10X T4 ligation buffer	1.0 µl
Linearized Recipient Vector EcoRI/PstI (Chloramphenicol)	0.5 µl
T4 DNA Ligase (5Weiss/µl)	0.5µl

9.3. TRANSFORMATION

Materials

j. LB Chloramphenicol 34 µg/mL agar plates, Teknova
k. DH5a competent cells, Invitrogen, Cat 18265-017
l. SOC (Recovery Medium), Lucigen, Cat No. F98226
m. 1.5 mL tube
n. Vortex
o. Pipet and tips
p. Ice bucket and ice
q. Water bath (42°C)
r. Incubator (37°C)

Method

r. Transform ligated DNA into E.coli DH5a chemically competent cells
s. Turn on incubator-shaker at 37°C.
t. Turn on incubator for plates at 37°C.
u. Set up water bath at 42°C.
v. Bring to room temperature S.O.C medium.
w. Bring LB plates supplemented with appropriate antibiotic at room temperature.

x. Thaw competent cells on ice.
y. Aliquots competent cells in as many tubes as needed.
z. Add 5 µl ligation mix to 50 µl competent cells to DNA and swirl gently to mix.
aa. Incubate on ice for 20 minutes
bb. Heat shock at 42°C in heat block for 30 seconds. Quickly return to ice and let it sit for 2 min.
cc. Add 200 µl of 18-25°C SOC medium and transfer mixture to 15mL Falcon tube
dd. Incubate in shaker at 37°C, 225 rpm for 30 min
ee. Plate 2 volumes (50 µL and ~100µL) of the mixture onto 2 different plates of LB agar plates supplemented with tetracycline12.5 µg/mL
ff. Incubate plates at 37°C overnight
gg. Count colonies and estimate transformation efficiency
hh. Initiate cultures in LB with the appropriate antibiotic from individual clones to analyze clones

9.4. TRANSFORMATION RESULTS

Description [ligation]	**Number of colonies (50 µL volume plating)**	**Number of clones analyzed by digestion**	**Clones Designation**
Control: Recipient vector [EcoRI/PstI]	0		
Ligation: Recipient vector pSB1C3+ F1_F2 each under control of promoter BBa_J23100	>100	2	CCA-66 CCA-67
Ligation: Recipient vector pSB1C3+ F1_F2 each under control of promoter BBa_J23101	>100	2	CCA-68 CCA-69
Ligation: Recipient vector pSB1C3+ F1_F2 each under control of promoter BBa_J23110	>100	2	CCA-70 CCA-71

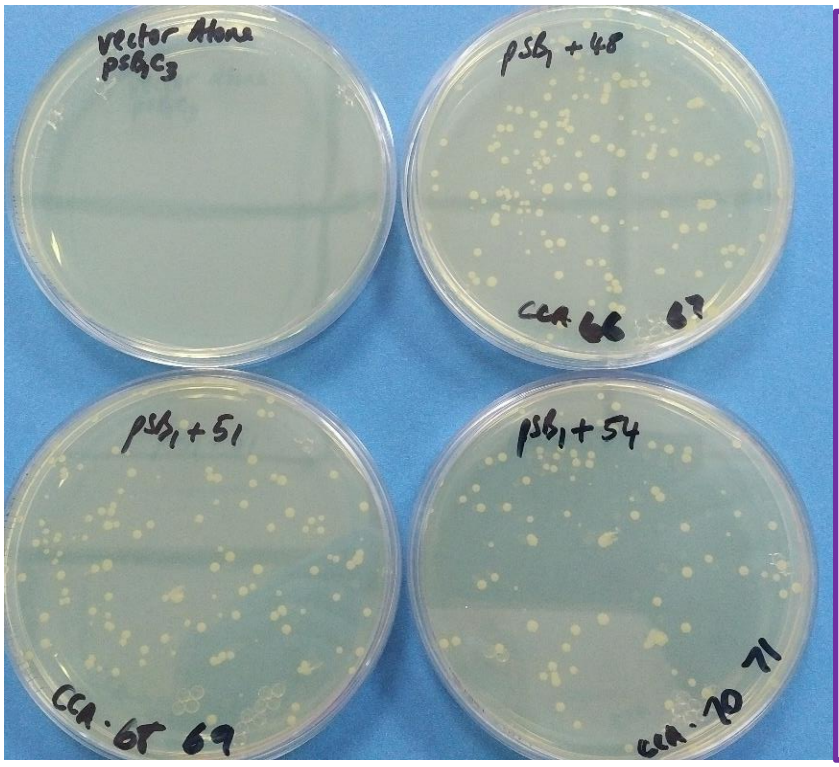


Figure. LB-Chloramphenicol plates with E.coli DH5a cells transformed with product of ligation of pSB1C3 ligated to Fluorene-1 + Fluroene-2 (XbaI/PstI) under the control of the constitutive promoter BBa_J23100 [100] or BBa_J23101 [101] or BBa_J23110[110].

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

Fluorene-1= Promoter_RBS_Synthetic flnB, dbfA1, dbfA2
Fluorene-2= Promoter_RBS_Synthetic flnE, flnD1, ORF16, flnC

9.5. CLONE VERIFICATION

Clone verification is done by digestion. Cultures are set up to extract DNA.

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

f. Grow selected number of colonies in 3 mL LB medium supplemented with tetracycline (12.5µg/mL) overnight at 37°C in the incubator/shaker at 220 rpm.
g. Spin down 1.5 mL of culture from each culture and isolate plasmid DNA using the Zymo Research DNA Isolation kit.
h. DNA preparation is resuspended in a final volume of **35 µL** reagent grade water.
i. Store remaining 1mL of culture for glycerol stock preparation of sequence confirmed clones.
j. Check clones by digestion.

Clones
Analysis of Individual clones: CCA_66 and CCA_67
BBa_J23100_RBS_F1_Terminator + BBa_J23100_RBS_F2_Terminator in vector pSB1C3

Analysis of Individual clones: CCA_68 and CCA_69
BBa_J23101_RBS_F1_Terminator +BBa_J23101_RBS_F2_Terminator in vector pSB1C3

Analysis of Individual clones: CCA_70 and CCA_71
BBa_J23110_RBS_F1_Terminator + BBa_J23110_RBS_F2_Terminator in vector pSB1C3

F1: Synthetic flnB, dbfA1, dbfA2
F2: Synthetic flnE, flnD1, ORF16, flnC

Set-up Digestion for Clone Verification
Date: 7-Aug-2017

l. Set up digestion as shown below.
m. This is a 1% gel agarose gel, TAE, prepared with Sybr green dye.
n. Digest clones with EcorRI/PstI
o. Set-up restriction digestion (20µl reaction) in a 1.5 mL tube
p. Turn on water bath at 37°C
q. Add the reagents in the order and with volume described in the table below.
r. Spin the tube briefly for 15 seconds at 10,000 rpm
s. Incubate the tube at 37°C for 15 min
t. The reaction already have a loading buffer
u. Load the digestion reaction on a 1% Agarose gel, TAE
v. Set up the power for electrophoresis to 120 V and let it run for ~ 1 hour.

Component	Volume	**Final condition**
Reagent grade water	15.0 µl	
10X BufferFastDigest Green Buffer	1.5 µl	1 X
Plasmid miniprep	1.5 µl	~0.2-0.4 µg
Restriction Enzyme 1	1.0 µl	

Restriction Enzyme 2	1.0 µl	

Expected Fragments Size for Verification

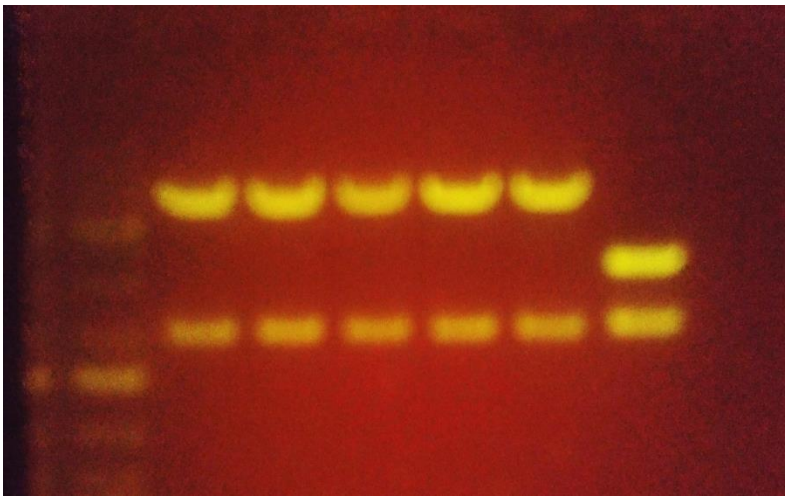
Fluorene: EcoRI/PstI digestion:
2 fragments should be observed: the vector pSB1C3 and F1+F2 (3157bp+3468 bp)

F1_Terminator: XbaI/Pst1: 3107 bp
Promoter_RBS_F1_Terminator: 3175 bp
Promoter_RBS_F1_Terminator: 3157 bp EcoRI/SpeI

F2_Terminator: XbaI/Pst1: 3433 bp
Promoter_RBS_F_Terminator2: 3501 bp
Promoter_RBS_F2_Terminator: 3468 bp EcoRI/SpeI

Results
Clones are ok except for CCA-71.
Clone CCA-66, CCA68, and CCA70 will be sent out for sequencing for verification.

CCA-66 67 68 69 70 71



9.6. VERIFICATION BY SEQUENCING OF FULL LENGTH CLONE F1_F2

An aliquot of the DNA plasmid mini-prep was sent out for sequencing.
All files are attached below.
The data showed that the sequence of the clones was correct.

10- GLYCEROL STOCK

Date: 14-AUG-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 appropriate antibiotics 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 pathway Phenanthrene 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
CCA-1009	14-Aug-17	CCA-23	Promoter BBa_J23100 /RBS_BBa_B0034 EcoRI/SpeI + Phenanthrene insert 1_Ter_BBa_B0015 XbaI/PstI + pSB1C3 EcoRI/PstI	E.coli DH5a	Chloramphenicol	pSB1C3
CCA-1010	14-Aug-17	CCA-26	Promoter BBa_J23101 /RBS_BBa_B0034 EcoRI/SpeI + Phenanthrene insert 1_Ter_BBa_B0015 XbaI/PstI + pSB1C3 EcoRI/PstI	E.coli DH5a	Chloramphenicol	pSB1C3
CCA-1011	14-Aug-17	CCA-29	Promoter BBa_J23110 /RBS_BBa_B0034 EcoRI/SpeI + Phenanthrene insert 1_Ter_BBa_B0015 XbaI/PstI + pSB1C3 EcoRI/PstI	E.coli DH5a	Chloramphenicol	pSB1C3
CCA-1012	14-Aug-17	CCA-30	Fluorene insert 1_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23100_BBa_B0034 EcoRI/SpeI+ pSB1C3 EcoRI/PstI	E.coli DH5a	Chloramphenicol	pSB1C3
CCA-1013	14-Aug-17	CCA-32	Fluorene insert 1_Ter_BBa_B0015 XbaI/PstI + Promoter 14-Aug-17BBa_J23101_BBa_B0034 EcoRI/SpeI+ pSB1C3 EcoRI/PstI	E.coli DH5a	Chloramphenicol	pSB1C3
CCA-1014	14-Aug-17	CCA-34	Fluorene insert 1_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23110_BBa_B0034 EcoRI/SpeI+ pSB1C3 EcoRI/PstI	E.coli DH5a	Chloramphenicol	pSB1C3
CCA-1015	14-Aug-17	CCA-36	Fluorene insert 2_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23100_BBa_B0034 EcoRI/SpeI+ pSB1C3 EcoRI/PstI	E.coli DH5a	Chloramphenicol	pSB1C3
CCA-1035	14-Aug-17	CCA-38	Fluorene insert 2_Ter_BBa_B0015 XbaI/PstI + Promoter	E.coli DH5a	Chloramphenicol	pSB1C3

			BBa_J23101_BBa_B0034 EcoRI/SpeI+ pSB1C3 EcoRI/PstI			
CCA-1016	14-Aug-17	CCA-40	Fluorene insert 2_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23110_BBa_B0034 EcoRI/SpeI+ pSB1C3 EcoRI/PstI	E.coli DH5a	Chloramphenicol	pSB1C3
CCA-1017	14-Aug-17	CCA-42	Phenanthrene insert 2_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23100_BBa_B0034 EcoRI/SpeI+ pSB1C3 EcoRI/PstI	E.coli DH5a	Chloramphenicol	pSB1C3
CCA-1018	14-Aug-17	CCA-44	Phenanthrene insert 2_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23101_BBa_B0034 EcoRI/SpeI+ pSB1C3 EcoRI/PstI	E.coli DH5a	Chloramphenicol	pSB1C3
CCA-1019	14-Aug-17	CCA-46	Phenanthrene insert 2_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23110_BBa_B0034 EcoRI/SpeI+ pSB1C3 EcoRI/PstI	E.coli DH5a	Chloramphenicol	pSB1C3
CCA-1020	14-Aug-17	CCA-48	CCA-30 [Fluorene insert 1_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23100_BBa_B0034 EcoRI/SpeI] as EcoRI/SpeI CCA-36 [Fluorene insert 2_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23100_BBa_B0034 EcoRI/SpeI] as XbaI/PstI Vector pSB3T5 as EcoRI/PstI	E.coli DH5a	Tetracycline	pSB3T5
CCA-1021	14-Aug-17	CCA-51	CCA-30 [Fluorene insert 1_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23101_BBa_B0034 EcoRI/SpeI] as EcoRI/SpeI CCA-38 [Fluorene insert 2_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23101_BBa_B0034 EcoRI/SpeI] as XbaI/PstI Vector pSB3T5 as EcoRI/PstI	E.coli DH5a	Tetracycline	pSB3T5
CCA-1022	14-Aug-17	CCA-54	CCA-30 [Fluorene insert 1_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23110_BBa_B0034 EcoRI/SpeI] as EcoRI/SpeI CCA-40 [Fluorene insert 2_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23110_BBa_B0034 EcoRI/SpeI] as XbaI/PstI Vector pSB3T5 as EcoRI/PstI	E.coli DH5a	Tetracycline	pSB3T5
CCA-1023	14-Aug-17	CCA-57	CCA-23 [Promoter BBa_J23100 /RBS_BBa_B0034 EcoRI/SpeI + Phenanthrene insert 1_Ter_BBa_B0015 XbaI/PstI] as SpeI/PstI CCA-42 [Phenanthrene insert 2_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23100_BBa_B0034 EcoRI/SpeI] as XbaI/PstI	E.coli DH5a	Chloramphenicol	pSB1C3
CCA-1024	14-Aug-17	CCA-60	CCA-26 [Promoter BBa_J23101 /RBS_BBa_B0034 EcoRI/SpeI + Phenanthrene insert 1_Ter_BBa_B0015 XbaI/PstI] as SpeI/PstI CCA-44 [Phenanthrene insert 2_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23101_BBa_B0034 EcoRI/SpeI] as XbaI/PstI	E.coli DH5a	Chloramphenicol	pSB1C3
CCA-1025	14-Aug-17	CCA-64	CCA-29 [Promoter BBa_J23110 /RBS_BBa_B0034 EcoRI/SpeI + Phenanthrene insert 1_Ter_BBa_B0015 XbaI/PstI] as SpeI/PstI CCA-46 [Phenanthrene insert 2_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23110_BBa_B0034 EcoRI/SpeI] as XbaI/PstI	E.coli DH5a	Chloramphenicol	pSB1C3
CCA-1026	14-Aug-17	CCA-48	CCA-30 [Fluorene insert 1_Ter_BBa_B0015 XbaI/PstI + Promoter	E.coli BL-21	Tetracycline	pSB3T5

			BBa_J23100_BBa_B0034 EcoRI/SpeI] as EcoRI/SpeI CCA-36 [Fluorene insert 2_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23100_BBa_B0034 EcoRI/SpeI] as XbaI/PstI Vector pSB3T5 as EcoRI/PstI			
CCA-1027	14-Aug-17	CCA-51	CCA-32 [Fluorene insert 1_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23101_BBa_B0034 EcoRI/SpeI] as EcoRI/SpeI CCA-38 [Fluorene insert 2_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23101_BBa_B0034 EcoRI/SpeI] as XbaI/PstI Vector pSB3T5 as EcoRI/PstI	E.coli BL-21	Tetracycline	pSB3T5
CCA-1028	14-Aug-17	CCA-54	CCA-34 [Fluorene insert 1_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23110_BBa_B0034 EcoRI/SpeI] as EcoRI/SpeI CCA-40 [Fluorene insert 2_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23110_BBa_B0034 EcoRI/SpeI] as XbaI/PstI Vector pSB3T5 as EcoRI/PstI	E.coli BL-21	Tetracycline	pSB3T5
CCA-1029	14-Aug-17	CCA-57	CCA-23 [Promoter BBa_J23100 /RBS_BBa_B0034 EcoRI/SpeI + Phenanthrene insert 1_Ter_BBa_B0015 XbaI/PstI] as SpeI/PstI CCA-42 [Phenanthrene insert 2_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23100_BBa_B0034 EcoRI/SpeI] as XbaI/PstI	E.coli BL-21	Chloramphenicol	pSB1C3
CCA-1030	14-Aug-17	CCA-60	CCA-26 [Promoter BBa_J23101 /RBS_BBa_B0034 EcoRI/SpeI + Phenanthrene insert 1_Ter_BBa_B0015 XbaI/PstI] as SpeI/PstI CCA-44 [Phenanthrene insert 2_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23101_BBa_B0034 EcoRI/SpeI] as XbaI/PstI	E.coli BL-21	Chloramphenicol	pSB1C3
CCA-1031	14-Aug-17	CCA-64	CCA-29 [Promoter BBa_J23110 /RBS_BBa_B0034 EcoRI/SpeI + Phenanthrene insert 1_Ter_BBa_B0015 XbaI/PstI] as SpeI/PstI CCA-46 [Phenanthrene insert 2_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23110_BBa_B0034 EcoRI/SpeI] as XbaI/PstI	E.coli BL-21	Chloramphenicol	pSB1C3
CCA-1032	14-Aug-17	CCA-48 + CCA-57		E.coli BL-21	Chloramphenicol, Tetracycline	pSB1C3, pSB3T5
CCA-1033	14-Aug-17	CCA-51 + CCA-60		E.coli BL-21	Chloramphenicol, Tetracycline	pSB1C3, pSB3T5
CCA-1034	14-Aug-17	CCA-54 + CCA-64		E.coli BL-21	Chloramphenicol, Tetracycline	pSB1C3, pSB3T5
CCA-1036	14-Aug-17			E.coli BL-21	Tetracycline	pSB3T5

CCA-1037				E.coli BL-21	Chloramphenicol	pSB1C3
CCA-1038	14-Aug-17			E.coli BL-21	Chloramphenicol, Tetracycline	pSB1C3, pSB3T5
CCA-1039	1-Sep-17	CCA-66	CCA-30 [Fluorene insert 1_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23100_BBa_B0034 EcoRI/SpeI] as EcoRI/SpeI CCA-36 [Fluorene insert 2_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23100_BBa_B0034 EcoRI/SpeI] as XbaI/PstI Vector pSB3T5 as EcoRI/PstI	E.coli DH5a	Chloramphenicol	pSB1C3
CCA-1040	1-Sep-17	CCA-68	CCA-32 [Fluorene insert 1_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23101_BBa_B0034 EcoRI/SpeI] as EcoRI/SpeI CCA-38 [Fluorene insert 2_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23101_BBa_B0034 EcoRI/SpeI] as XbaI/PstI Vector pSB3T5 as EcoRI/PstI	E.coli DH5a	Chloramphenicol	pSB1C3
CCA-1041	1-Sep-17	CCA-70	CCA-34 [Fluorene insert 1_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23110_BBa_B0034 EcoRI/SpeI] as EcoRI/SpeI CCA-40 [Fluorene insert 2_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23110_BBa_B0034 EcoRI/SpeI] as XbaI/PstI Vector pSB3T5 as EcoRI/PstI	E.coli DH5a	Chloramphenicol	pSB1C3

11- SEQUENCING FILES

Primers:

Name	Description	Sequence	%GC	Tm	Length
BBa_G00100	Forward primer for sequencing/amplifying BioBrick parts (VF2)	tgccacctgacgtctaagaa	50	60C	20
BBa_G00101	Reverse primer for sequencing/amplifying BioBrick parts (VR)	attaccgcctttgagtgagc	50	60C	20

Sequence verification

Fluorene clones CCA-48, CCA-51, and CCA-54.

Sequence Alignment of clones CCA-58, CCA-51, and CCA-54.

```
CLUSTAL O(1.2.4) multiple sequence alignment
Forward sequence

54      TGCGAGAAGGCCCACCCGTGAAGGTGAGCCAGTGAGTTGATTGCTACGTAATTAGTTAGT
48      --GAAGAAGGCCCACCCGTGAAGGTGAGCCAGTGAGTTGATTGCTACGTAATTAGTTAGT
51      --------------CCCGTGAAGGTGAGCCAGTGAGTTGATTGCTACGTAATTAGTTAGT
                     ******************************************

54      TAGCCCTTAGTGACTCGAATTCGCGGCCGCTTCTAGATTTACGGCTAGCTCAGTCCTAGG
48      TAGCCCTTAGTGACTCGAATTCGCGGCCGCTTCTAGATTGACGGCTAGCTCAGTCCTAGG
51      TAGCCCTTAGTGACTCGAATTCGCGGCCGCTTCTAGATTTACAGCTAGCTCAGTCCTAGG
        ************************************ ** *****************

54      TACAATGCTAGCAAAGAGGAGAAAACTAGATGTCCGAATCAGGGGGTGGGACTGTTGCTA
48      TACAGTGCTAGCAAAGAGGAGAAAACTAGATGTCCGAATCAGGGGGTGGGACTGTTGCTA
51      TATTATGCTAGCAAAGAGGAGAAAACTAGATGTCCGAATCAGGGGGTGGGACTGTTGCTA
        **   ********************************************************

54      CCGCACGTCAGCGCCAGTTGGTGGAACGTGCATTGGGCGAGTGGCAAGGAGAAGTGGCGG
48      CCGCACGTCAGCGCCAGTTGGTGGAACGTGCATTGGGCGAGTGGCAAGGAGAAGTGGCGG
51      CCGCACGTCAGCGCCAGTTGGTGGAACGTGCATTGGGCGAGTGGCAAGGAGAAGTGGCGG
        ************************************************************

54      GTCGCGTAATTGTGGTAACAGGTGGGGCTCGCGGGATCGGTCGCAGTTTATGTGAAGGTC
48      GTCGCGTAATTGTGGTAACAGGTGGGGCTCGCGGGATCGGTCGCAGTTTATGTGAAGGTC
51      GTCGCGTAATTGTGGTAACAGGTGGGGCTCGCGGGATCGGTCGCAGTTTATGTGAAGGTC
        ************************************************************
```

```
54      TTTTACGCGCAGGTGCCAAGGTCGTGGCCGCTGATTTAACCTGGGACGACGCCGATGACT
48      TTTTACGCGCAGGTGCCAAGGTCGTGGCCGCTGATTTAACCTGGGACGACGCCGATGACT
51      TTTTACGCGCAGGTGCCAAGGTCGTGGCCGCTGATTTAACCTGGGACGACGCCGATGACT
        ************************************************************

CLUSTAL O(1.2.4) multiple sequence alignment
Reverse sequence
48R      ATTNCNCGGNGCCGCGTGN-GNTNNACTACTAGGCNTNNATTTTCNNCTTTGGGACGAGG
51R      -------------------------TCTACNGGATTTTTTTTTCCCNATTTGGGACGAGG
54R      ----------GCCGCGNGNGGAAAACTACAAGGGATTTATTTTCGCAANNTGGGACGAGG
                                    *   *   ***       **********

48R      ACGCTATTCCCCTGGAGGATTATCTGGGGGCAGATCCCTTATGGTACTTANACTTAGCCT
51R      ACGCTATTCCCCTGTAGGATTATCTGGGGGCAGATCCCT-TATGGNCTGAGACTTAGCCT
54R      ACGCTATTCCCCTGGTGGATTATCTGGGGGCATATCCTT-ATGGNACTTAGACTTAGCCT
         **************  *************** **** *    *  ** * *********

48R      TCGAAGCACCACTGGGGGGGTTAGAGGTGATTGGTCCGACGATGAAATTTCGCATTAAGG
51R      TCGAAGCACCACTGGGGGGGNTAGAGGTGATTGGACCGACGATGAAATTTCGCATTAAGG
54R      TCGAAGCACCACTGGGGGGGTTAGAGGTGATTGGACCGACGATGAAATTTCGCATTAAGG
         ******************** ************* ************************

48R      GCAATTGGAAACTTGCGGCGGAGAACTTCGCCGGANACGACTACCACGGTTTGTACACCC
51R      GCATTTGTAAACTTGCGGCGGAAAACTTCGCCGGAGACGACTACCACGATTTGTACACCC
54R      GCAATTGGAAACTTGCGGCGGAGAACTTCGCCGGAGACGACTACCACGTTTTGTACACCC
         *** *** ************** ************ ************ ************

48R      ATGGGGCAACTTTCCAGATTGGGTTCCTGCCCGATTACGACACACTTGGNGATTACATCG
51R      ATGGGTCAACTTTCCAGATTGGCTTCCTGCCCGATTACGACACACTTGGNGATTACATCG
54R      ATGGGTCAGCTTTCCAGATTGGCTTCCTGCCCGATTACGACACACTTGGCGATTACATCG
         ***** ** ************** ************************* **********

48R      CTCATTTTGACCACGGACACGGAATGGGCGACATTGGTAAGCCTGGACGCGCCTATCAAA
51R      CTCATTTTGACCACGGACACGGAATGGGCGACATTGGTAAGCCTGGACGCGCCTATCAAA
54R      CTCATTTTGACCACGGACACGGAATGGGCGACATTGGTAAGCCTGGACGCGCCTATCAAA
         ************************************************************

48R      ACGACGTGGGGATGGCGCAATTCTTGGGCCCTGAGGCTATCGAATATGTTAACGCGGTGC
51R      ACGACGAGGGGATGGCGCAATTCTTGGGCCCTGAGGCTATCGAATATGTTAACGCGGTGC
54R      ACGACGTGGGGATGGCGCAATTCTTGGGCCCTGAGGCTATCGAATATGTTAACGCGGTGC
         ****** **************************************************

48R      ATGAACGTTTGAAAGCCCGTGTCTCACCGTTGCAAGCGGAGATGCACGGCTTAGGGCAAG
51R      ATGAACGTTTGAAAGCCCGTGTCTCACCGTTGCAAGCGGAGATGCACGGCTTAGGGCAAG
54R      ATGAACGTTTGAAAGCCCGTGTCTCACCGTTGCAAGCGGAGATGCACGGCTTAGGGCAAG
         ************************************************************
```

Translation clone CCA-48 (forward)

>48-BBaG00100-F 1256 30 1087 0.05

XXXXXEEGPPVKVSQ-VDCYVIS-LALSDSNSRPLLD-RLAQS-VQC-QRGEN-MSESGG
GTVATARQRQLVERALGEWQGEVAGRVIVVTGGARGIGRSLCEGLLRAGAKVVAADLTWD
DADDFRKQLESDGSGMAVDMDITDDDALDAARDAVIDRFGTVDVLVNNASLVSETLFPPT

GHRNTLDTTDRDWEVMFGVNVFGTLKAIRRFIEPMRAQQRGSIVNVVSSGVLAVAAGGGY
HGLRPWTVEMPYQATKAAVMALTFYLAEEVRGDGVAVNAIMPGHTRASWFDATARAFNEQ
GIAYFMRPAIPEHLLPISLFLAAQDSAGASGRLYYVPEWNYDHGYGDYAXWQDHELPPDM
EEIYSRLEAATXXYEXXGVXXLPFDXXGXLXAXGMANLGXXXXXIXMXXXIXXXLXXX

Blast of clone CCA-48 (forward)
fluoren-9-ol dehydrogenase [Janibacter terrae]

Alignment statistics for match #1

Score	Expect	Method	Identities	Positives	Gaps
623 bits(1606)	0.0	Compositional matrix adjust.	305/306(99%)	305/306(99%)	0/306

```
Query  1    MSESGGGTVATARQRQLVERALGEWQGEVAGRVIVVTGGARGIGRSLCEGLLRAGAKVVA  60
            MSESGGGTVATARQRQLVERALGEWQGEVAGRVIVVTGGARGIGRSLCEGLLRAGAKVVA
Sbjct  1    MSESGGGTVATARQRQLVERALGEWQGEVAGRVIVVTGGARGIGRSLCEGLLRAGAKVVA  60

Query  61   ADLTWDDADDFRKQLESDGSGMAVDMDITDDDALDAARDAVIDRFGTVDVLVNNASLVSE  120
            ADLTWDDADDFRKQLESDGSGMAVDMDITDDDALDAARDAVIDRFGTVDVLVNNASLVSE
Sbjct  61   ADLTWDDADDFRKQLESDGSGMAVDMDITDDDALDAARDAVIDRFGTVDVLVNNASLVSE  120

Query  121  TLFPPTGHRNTLDTTDRDWEVMFGVNVFGTLKAIRRFIEPMRAQQRGSIVNVVSSGVLAV  180
            TLFPPTGHRNTLDTTDRDWEVMFGVNVFGTLKAIRRFIEPMRAQQRGSIVNVVSSGVLAV
Sbjct  121  TLFPPTGHRNTLDTTDRDWEVMFGVNVFGTLKAIRRFIEPMRAQQRGSIVNVVSSGVLAV  180

Query  181  AAGGGYHGLRPWTVEMPYQATKAAVMALTFYLAEEVRGDGVAVNAIMPGHTRASWFDATA  240
            AAGGGYHGLRPWTVEMPYQATKAAVMALTFYLAEEVRGDGVAVNAIMPGHTRASWFDATA
Sbjct  181  AAGGGYHGLRPWTVEMPYQATKAAVMALTFYLAEEVRGDGVAVNAIMPGHTRASWFDATA  240

Query  241  RAFNEQGIAYFMRPAIPEHLLPISLFLAAQDSAGASGRLYYVPEWNYDHGYGDYAXWQDH  300
            RAFNEQGIAYFMRPAIPEHLLPISLFLAAQDSAGASGRLYYVPEWNYDHGYGDYA WQDH
Sbjct  241  RAFNEQGIAYFMRPAIPEHLLPISLFLAAQDSAGASGRLYYVPEWNYDHGYGDYAAWQDH  300

Query  301  ELPPDM  306
            ELPPDM
Sbjct  301  ELPPDM  306
```

Translation
>48-BBaG00100-R 1274 289 277 0.05
XXXXXXXLXXXXAAXXXLLGXXFXLWDEDAIPLEDYLGADPLWYLXLAFEAPLGGLEVIG
PTMKFRIKGNWKLAAENFAGXDYHGLYTHGATFQIGFLPDYDTLXDYIAHFDHGHGMGDI
GKPGRAYQNDVGMAQFLGPEAIEYVNAVHERLKARVSPLQAEMHGLGQGNIFPNLSWIKF
GVFHVFGLFQXHPRGPGEIEVWXTALXDRDAPXSXKDXAXXXMSQXXAXAGIFXXDDGEN
FEXITEXXXGVXSHTXDFHYAMXXGHEGXXXXKXTPVHLXXXYSEXNXRISXGIGXELMT
XSXNKXXXPSXXXXDHLLNEFTXXXXXXXVLXXQXXWXXXXXXXXX-SXXXXTSLXXAHX

Blast
aromatic ring-hydroxylating dioxygenase subunit alpha [Janibacter terrae]
Sequence ID: WP_032491530.1Length: 443Number of Matches: 1

Alignment statistics for match #1

Score	Expect	Method	Identities	Positives	Gaps
328 bits(842)	1e-109	Compositional matrix adjust.	160/180(89%)	161/180(89%)	0/180

```
Query  1    PTMKFRIKGNWKLAAENFAGXDYHGLYTHGATFQIGFLPDYDTLXDYIAHFDHGHGMGDI  60
            PTMKFRIK NWKLAAENFAG DYH LYTHG+ FQIGFLPDYDTL DYIAHFDHGHGMGDI
```

```
Sbjct  198  PTMKFRIKANWKLAAENFAGDDYHVLYTHGSAFQIGFLPDYDTLGDYIAHFDHGHGMGDI  257

Query  61   GKPGRAYQNDVGMAQFLGPEAIEYVNAVHERLKARVSPLQAEMHGLGQGNIFPNLSWIKF  120
            GKPGRAYQNDVGMAQFLGPEAIEYVNAVHERLKARVSPLQAEMHGLGQGNIFPNLSWIKF
Sbjct  258  GKPGRAYQNDVGMAQFLGPEAIEYVNAVHERLKARVSPLQAEMHGLGQGNIFPNLSWIKF  317

Query  121  GVFHVFGLFQXHPRGPGEIEVWXTALXDRDAPXSXKDXAXXXMSQXXAXAGIFXXDDGEN  180
            GVFHVFGLFQ HPRGPGEIEVW TAL DRDAP S KD A   MSQ  A AGIF  DDGEN
Sbjct  318  GVFHVFGLFQWHPRGPGEIEVWQTALFDRDAPQSVKDVARTQMSQENAAAGIFGQDDGEN  377
```

DNA sequences of clones CCA-48, CCA-51, and CCA-54 obtained using primers BBaG00100-F and BBaG00100-R

```
>48-BBaG00100-F
GAAGAAGGCCCACCCGTGAAGGTGAGCCAGTGAGTTGATTGCTACGTAATTAGTTAGTTAGCCCTTAGTGACTCGAATTCGCGGCCGCTTCTAGATTGACGGCT
AGCTCAGTCCTAGGTACAGTGCTAGCAAAGAGGAGAAAACTAGATGTCCGAATCAGGGGGTGGGACTGTTGCTACCGCACGTCAGCGCCAGTTGGTGGAACGTG
CATTGGGCGAGTGGCAAGGAGAAGTGGCGGGTCGCGTAATTGTGGTAACAGGTGGGGCTCGCGGGATCGGTCGCAGTTTATGTGAAGGTCTTTTACGCGCAGGT
GCCAAGGTCGTGGCCGCTGATTTAACCTGGGACGACGCCGATGACTTCCGCAAACAATTAGAGTCCGACGGCTCTGGTATGGCCGTAGATATGGATATTACAGA
CGATGATGCCTTGGACGCTGCCCGTGACGCAGTAATCGACCGCTTCGGAACCGTTGATGTCTTGGTGAATAACGCTTCGCTGGTCTCTGAGACTTTGTTTCCAC
CAACGGGGCACCGTAATACCCTGGACACGACAGATCGCGACTGGGAGGTAATGTTTGGTGTGAATGTCTTTGGAACACTTAAGGCGATTCGTCGCTTCATCGAG
CCAATGCGCGCTCAACAGCGCGGTTCGATTGTCAACGTGGTAAGCAGTGGCGTCCTTGCAGTCGCAGCTGGCGGGGGATACCATGGCTTGCGTCCATGGACCGT
TGAGATGCCCTATCAGGCTACTAAAGCAGCTGTCATGGCTCTTACATTCTACTTGGCCGAAGAGGTGCGCGGCGATGGGGTGGCGGTCAATGCTATCATGCCTG
GTCACACCCGCGCTTCTTGGTTTGATGCGACCGCTCGTGCCTTTAATGAGCAGGGGATCGCATACTTCATGCGCCCTGCTATTCCCGAGCACTTGCTTCCTATC
TCCTTGTTCCTTGCAGCGCAGGACTCCGCTGGCGCCTCTGGGCGTCTTTACTATGTGCCCGAGTGGAACTACGACCACGGGTACGGCGATTATGCGGNGTGGCA
GGATCATGAATTACCACCGGACATGGAGGAAATCTATTCGCGTCTGGAAGCAGCAACGCCNANTTATGAACGNNCAGGTGTANNNCNNTTGCCGTTTGATGCNN
AAGGAACNTTGNNTGCCNNAGGAATGGCTAATCTTGGAANANNGAANNNTNGGATTNCAATGNNTNNNNNNATAANTNNNAANTTAANCCGNNNA
>51-BBaG00100-F
CCCGTGAAGGTGAGCCAGTGAGTTGATTGCTACGTAATTAGTTAGTTAGCCCTTAGTGACTCGAATTCGCGGCCGCTTCTAGATTTACAGCTAGCTCAGTCCTA
GGTATTATGCTAGCAAAGAGGAGAAAACTAGATGTCCGAATCAGGGGGTGGGACTGTTGCTACCGCACGTCAGCGCCAGTTGGTGGAACGTGCATTGGGCGAGT
GGCAAGGAGAAGTGGCGGGTCGCGTAATTGTGGTAACAGGTGGGGCTCGCGGGATCGGTCGCAGTTTATGTGAAGGTCTTTTACGCGCAGGTGCCAAGGTCGTG
GCCGCTGATTTAACCTGGGACGACGCCGATGACTTCCGCAAACAATTAGAGTCCGACGGCTCTGGTATGGCCGTAGATATGGATATTACAGACGATGATGCCTT
GGACGCTGCCCGTGACGCAGTAATCGACCGCTTCGGAACCGTTGATGTCTTGGTGAATAACGCTTCGCTGGTCTCTGAGACTTTGTTTCCACCAACGGGGCACC
GTAATACCCTGGACACGACAGATCGCGACTGGGAGGTAATGTTTGGTGTGAATGTCTTTGGAACACTTAAGGCGATTCGTCGCTTCATCGAGCCAATGCGCGCT
CAACAGCGCGGTTCGATTGTCAACGTGGTAAGCAGTGGCGTCCTTGCAGTCGCAGCTGGCGGGGGATACCATGGCTTGCGTCCATGGACCGTTGAGATGCCCTA
TCAGGCTACTAAAGCAGCTGTCATGGCTCTTACATTCTACTTGGCCGAAGAGGTGCGCGGCGATGGGGTGGCGGTCAATGCTATCATGCCTGGTCACACCCGCG
CTTCTTGGTTTGATGCGACCGCTCGTGCCTTTAATGAGCAGGGGATCGCATACTTCATGCGCCCTGCTATTCCCGAGCACTTGCTTCCTATCTCCTTGTTCCTT
GCAGCGCAGGACTCCGCTGGCGCCTCTGGGCGTCTTTACTATGTGCCCGANTGGAACTACGACCACGGGTACGGCGATTATGCGGNGTGGCAGGATCATG

>54-BBaG00100-F 1279 19 1006 0.05
TGCGAGAAGGCCCACCCGTGAAGGTGAGCCAGTGAGTTGATTGCTACGTAATTAGTTAGTTAGCCCTTAGTGACTCGAATTCGCGGCCGCTTCTAGATTTACGG
CTAGCTCAGTCCTAGGTACAATGCTAGCAAAGAGGAGAAAACTAGATGTCCGAATCAGGGGGTGGGACTGTTGCTACCGCACGTCAGCGCCAGTTGGTGGAACG
TGCATTGGGCGAGTGGCAAGGAGAAGTGGCGGGTCGCGTAATTGTGGTAACAGGTGGGGCTCGCGGGATCGGTCGCAGTTTATGTGAAGGTCTTTTACGCGCAG
GTGCCAAGGTCGTGGCCGCTGATTTAACCTGGGACGACGCCGATGACTTCCGCAAACAATTAGAGTCCGACGGCTCTGGTATGGCCGTAGATATGGATATTACA
GACGATGATGCCTTGGACGCTGCCCGTGACGCAGTAATCGACCGCTTCGGAACCGTTGATGTCTTGGTGAATAACGCTTCGCTGGTCTCTGAGACTTTGTTTCC
ACCAACGGGGCACCGTAATACCCTGGACACGACAGATCGCGACTGGGAGGTAATGTTTGGTGTGAATGTCTTTGGAACACTTAAGGCGATTCGTCGCTTCATCG
AGCCAATGCGCGCTCAACAGCGCGGTTCGATTGTCAACGTGGTAAGCAGTGGCGTCCTTGCAGTCGCAGCTGGCGGGGGATACCATGGCTTGCGTCCATGGACC
GTTGAGATGCCCTATCAGGCTACTAAAGCAGCTGTCATGGCTCTTACATTCTACTTGGCCGAAGAGGTGCGCGGCGATGGGGTGGCGGTCAATGCTATCATGCC
TGGTCACACCCGCGCTTCTTGGTTTGATGCGACCGCTCGTGCCTTTAATGAGCAGGGGATCGCATACTTCATGCGCCCTGCTATTCCCGAGCACTTGCTTCCTA
TCTCCTTGTTCCTTGCAGCGCAGGACTCCGCTGGGNCCTCTGGGGCGTCTTTACTATGNTGCCCGAGTGGAACTACCAACCNCGGGTTACGGCGAAT


>54-BBaG00100-F 1279 19 1006 0.05
```

TGCGAGAAGGCCCACCCGTGAAGGTGAGCCAGTGAGTTGATTGCTACGTAATTAGTTAGTTAGCCCTTAGTGACTCGAATTCGCGGCCGCTTCTAGATTTACGG
CTAGCTCAGTCCTAGGTACAATGCTAGCAAAGAGGAGAAAACTAGATGTCCGAATCAGGGGGTGGGACTGTTGCTACCGCACGTCAGCGCCAGTTGGTGGAACG
TGCATTGGGCGAGTGGCAAGGAGAAGTGGCGGGTCGCGTAATTGTGGTAACAGGTGGGGCTCGCGGGATCGGTCGCAGTTTATGTGAAGGTCTTTTACGCGCAG
GTGCCAAGGTCGTGGCCGCTGATTTAACCTGGGACGACGCCGATGACTTCCGCAAACAATTAGAGTCCGACGGCTCTGGTATGGCCGTAGATATGGATATTACA
GACGATGATGCCTTGGACGCTGCCCGTGACGCAGTAATCGACCGCTTCGGAACCGTTGATGTCTTGGTGAATAACGCTTCGCTGGTCTCTGAGACTTTGTTTCC
ACCAACGGGGCACCGTAATACCCTGGACACGACAGATCGCGACTGGGAGGTAATGTTTGGTGTGAATGTCTTTGGAACACTTAAGGCGATTCGTCGCTTCATCG
AGCCAATGCGCGCTCAACAGCGCGGTTCGATTGTCAACGTGGTAAGCAGTGGCGTCCTTGCAGTCGCAGCTGGCGGGGGATACCATGGCTTGCGTCCATGGACC
GTTGAGATGCCCTATCAGGCTACTAAAGCAGCTGTCATGGCTCTTACATTCTACTTGGCCGAAGAGGTGCGCGGCGATGGGGTGGCGGTCAATGCTATCATGCC
TGGTCACACCCGCGCTTCTTGGTTTGATGCGACCGCTCGTGCCTTTAATGAGCAGGGGATCGCATACTTCATGCGCCCTGCTATTCCCGAGCACTTGCTTCCTA
TCTCCTTGTTCCTTGCAGCGCAGGACTCCGCTGGGNCCTCTGGGGCGTCTTTACTATGNTGCCCGAGTGGAACTACCAACCNCGGGTTACGGCGAATNNN

>48R
ATTNCNCGGNGCCGCGTGNGNTNNACTACTAGGCNTNNATTTTCNNCTTTGGGACGAGGACGCTATTCCCCTGGAGGATTATCTGGGGGCAGATCCCTTATGGT
ACTTANACTTAGCCTTCGAAGCACCACTGGGGGGGTTAGAGGTGATTGGTCCGACGATGAAATTTCGCATTAAGGGCAATTGGAAACTTGCGGCGGAGAACTTC
GCCGGANACGACTACCACGGTTTGTACACCCATGGGGCAACTTTCCAGATTGGGTTCCTGCCCGATTACGACACACTTGGNGATTACATCGCTCATTTTGACCA
CGGACACGGAATGGGCGACATTGGTAAGCCTGGACGCGCCTATCAAAACGACGTGGGGATGGCGCAATTCTTGGGCCCTGAGGCTATCGAATATGTTAACGCGG
TGCATGAACGTTTGAAAGCCCGTGTCTCACCGTTGCAAGCGGAGATGCACGGCTTAGGGCAAGGAAATATTTTTCCAAATTTATCATGGATTAAATTCGGCGTG
TTTCACGTGTTTGGATTGTTTCAGNGGCATCCACGTGGGCCGGGGGAAATCGAAGTCTGGNAGACTGCCTTGNTTGATCGTGATGCTCCGCANAGTGNCAAGGA
CGTNGCTCNNANTCNNATGAGTCAGGANAANGCGNNTGCCGGAATCTTTGGNCANGACGACGGAGAAAATTTTGAACANATCACCGAATCNNNTCGNGGCGTTG
NCAGTCACACTCGNGACTTCCACTACGCTATGGGNNTGGGGCATGAGGGGNAANCCNTGANGAAGGTNACCCCTGTCCATTTANGNCCNNACTATTCCGAGCAN
AACCNTCGCATTTCTANCGGTATTGGCTGNGAGCTGATGACANCGTCGNANAACAAANNANTTGNCCCCTCGGANGNTANNNNCGACCACCTCCTTAACGAATT
TACGNNNNNACGNANANGANTNTN
>51R
TCTACNGGATTTTTTTTTCCCNATTTGGGACGAGGACGCTATTCCCCTGTAGGATTATCTGGGGGCAGATCCCTTATGGNCTGAGACTTAGCCTTCGAAGCACC
ACTGGGGGGGNTAGAGGTGATTGGACCGACGATGAAATTTCGCATTAAGGGCATTTGTAAACTTGCGGCGGAAAACTTCGCCGGAGACGACTACCACGATTTGT
ACACCCATGGGTCAACTTTCCAGATTGGCTTCCTGCCCGATTACGACACACTTGGNGATTACATCGCTCATTTTGACCACGGACACGGAATGGGCGACATTGGT
AAGCCTGGACGCGCCTATCAAAACGACGAGGGGATGGCGCAATTCTTGGGCCCTGAGGCTATCGAATATGTTAACGCGGTGCATGAACGTTTGAAAGCCCGTGT
CTCACCGTTGCAAGCGGAGATGCACGGCTTAGGGCAAGGAAATATTTTTCCAAATTTATCATGGATTAAATTCGGCGTGTTTCACGTGTTTGGATTGTTTCAGT
GGCATCCACGTGGGCCGGGGGAAATCGAAGTCTGGCAGACTGCCTTGTTTGATCGTGATGCTCCGCAAAGTGTCAAGGACGTNGCTCGTACTCAGATGAGTCAG
GAGAATGCGGCTGCCGGAATCTTTGGCCAGNACGACGGANAAAATTTTGAACAAATCACCGAATCAGCTCGNGGCGTTGNCAGNCAGACTCGCGACTTCNCTAC
GCTATGGGTTNGGGGCATGAGGGGAAATCCATGAGGAAGGTACCCTGNNCATTTANGTCCGCACTATTCCGAGCANAACCATCGCAATTTCTACCGNTATTGGN
NGGAGTTGANGACAACGCCAGGAGACCAAANTAATNGCCCCATCGGAGGATANTAT
>54R
GCCGCGNGNGGAAAACTACAAGGGATTTATTTTCGCAANNTGGGACGAGGACGCTATTCCCCTGGTGGATTATCTGGGGGCATATCCTTATGGNACTTAGACTT
AGCCTTCGAAGCACCACTGGGGGGGTTAGAGGTGATTGGACCGACGATGAAATTTCGCATTAAGGGCAATTGGAAACTTGCGGCGGAGAACTTCGCCGGAGACG
ACTACCACGTTTTGTACACCCATGGGTCAGCTTTCCAGATTGGCTTCCTGCCCGATTACGACACACTTGGCGATTACATCGCTCATTTTGACCACGGACACGGA
ATGGGCGACATTGGTAAGCCTGGACGCGCCTATCAAAACGACGTGGGGATGGCGCAATTCTTGGGCCCTGAGGCTATCGAATATGTTAACGCGGTGCATGAACG
TTTGAAAGCCCGTGTCTCACCGTTGCAAGCGGAGATGCACGGCTTAGGGCAAGGAAATATTTTTCCAAATTTATCATGGATTAAATTCGGCGTGTTTCACGTGT
TTGGATTGTTTCAGTGGCATCCACGTGGNNCGNNGGAAATCGAAGTCTGGCAGACTGCCTTGTTTGATCGTGATGCTCCGCANAGNGNCAAGGACGTTGCTCGT
ACTCAGATGANTCNGGANAATGCGGNTGCCGGAATCTTTGGNCAGNACGACNGAGAANTTNTGAACAAATCACCGAATCAGCTCNNNCGTTGNCAGTCANACTC
GCGACTTCCNCTACNNTATGGGTTTGGGGCATGANGGGGAAATCCATGAGGAAAGNTACCCTGGTCATTTAGGNCCGCACTATCCGAGNANGATNCATCGCAAT
TNC

Sequence verification

Phenanthrene clones CCA-57, CCA-60, and CCA-64.

Sequence Alignment of clones CCA-57, CCA-60, and CCA-64.

Clustal
Forward

```
64F                -------TCACGAGGCAGAATTTCAGATAAAAAAAATCCTTAGCTTTCGCTAAGGATGAT
57-BBaG00100-F     -GGCGTATCACGAGGCAGAATTTCAGATAAAAAAAATCCTTAGCTTTCGCTAAGGATGAT
```

```
60F                 AGGCGTATNACGAGGCAGAATTTCAGATAAAAAAAATCCTTAGCTTTCGCTAAGGATGAT
                           * ************************************************

64F                 TTCTGGAATTCGCGGCCGCTTCTAGATTTACGGCTAGCTCAGTCCTAGGTACAATGCTAG
57-BBaG00100-F      TTCTGGAATTCGCGGCCGCTTCTAGATTGACGGCTAGCTCAGTCCTAGGTACAGTGCTAG
60F                 TTCTGGAATTCGCGGCCGCTTCTAGATTTACAGCTAGCTCAGTCCTAGGTATTATGCTAG
                    *************************** ** ******************   ******

64F                 CAAAGAGGAGAAAACTAGATGGATACGCAACTTATCATTGATAACGCAGACGTTCCGGCG
57-BBaG00100-F      CAAAGAGGAGAAAACTAGATGGATACGCAACTTATCATTGATAACGCAGACGTTCCGGCG
60F                 CAAAGAGGAGAAAACTAGATGGATACGCAACTTATCATTGATAACGCAGACGTTCCGGCG
                    ************************************************************

64F                 ACTGCTGCCGCGACCTTTGAACGTCGTAGTCCTACAACCGGCGAATTAGTGACTCGCGCC
57-BBaG00100-F      ACTGCTGCCGCGACCTTTGAACGTCGTAGTCCTACAACCGGCGAATTAGTGACTCGCGCC
60F                 ACTGCTGCCGCGACCTTTGAACGTCGTAGTCCTACAACCGGCGAATTAGTGACTCGCGCC
                    ************************************************************

64F                 GCCGCCGCCAGCGTCGCTGACGCAATTGCAGCCGCTGACTCTGCTGCCGCAGCTTATCGT
57-BBaG00100-F      GCCGCCGCCAGCGTCGCTGACGCAATTGCAGCCGCTGACTCTGCTGCCGCAGCTTATCGT
60F                 GCCGCCGCCAGCGTCGCTGACGCAATTGCAGCCGCTGACTCTGCTGCCGCAGCTTATCGT
                    ************************************************************

64F                 TCCTGGAGCACTACTGGGCCCACCGAGCGCCGCCGCATCTTGTTGAAAGCCGCCGATTTA
57-BBaG00100-F      TCCTGGAGCACTACTGGGCCCACCGAGCGCCGCCGCATCTTGTTGAAAGCCGCCGATTTA
60F                 TCCTGGAGCACTACTGGGCCCACCGAGCGCCGCCGCATCTTGTTGAAAGCCGCCGATTTA
                    ************************************************************
```

Clustal Reverse sequences

```
57R       --ACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCCTGCATAACGCGAAGTAATCTTT
60R       CGACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCCTGCATAACGCGAAGTAATCTTT
64R       ----GACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCCTGCATAACGCGAAGTAATCTTT
              ********************************************************

57R       TCGGTTTTAAAGAAAAAGGGCAGGGTGGTGACACCTTGCCCTTTTTTGCCGGACTGCAGC
60R       TCGGTTTTAAAGAAAAAGGGCAGGGTGGTGACACCTTGCCCTTTTTTGCCGGACTGCAGC
64R       TCGGTTTTAAAGAAAAAGGGCAGGGTGGTGACACCTTGCCCTTTTTTGCCGGACTGCAGC
          ************************************************************

57R       GGCCGCTACTAGTATATAAACGCAGAAAGGCCCACCCGAAGGTGAGCCAGTGTGACTCTA
60R       GGCCGCTACTAGTATATAAACGCAGAAAGGCCCACCCGAAGGTGAGCCAGTGTGACTCTA
64R       GGCCGCTACTAGTATATAAACGCAGAAAGGCCCACCCGAAGGTGAGCCAGTGTGACTCTA
          ************************************************************

57R       GTAGAGAGCGTTCACCGACAAACAACAGATAAAACGAAAGGCCCAGTCTTTCGACTGAGC
60R       GTAGAGAGCGTTCACCGACAAACAACAGATAAAACGAAAGGCCCAGTCTTTCGACTGAGC
64R       GTAGAGAGCGTTCACCGACAAACAACAGATAAAACGAAAGGCCCAGTCTTTCGACTGAGC
          ************************************************************

57R       CTTTCGTTTTATTTGATGCCTGGTTATTAATGCAAATCATCACCACCACACACCTTCATA
60R       CTTTCGTTTTATTTGATGCCTGGTTATTAATGCAAATCATCACCACCACACACCTTCATA
64R       CTTTCGTTTTATTTGATGCCTGGTTATTAATGCAAATCATCACCACCACACACCTTCATA
          ************************************************************
```

```
57R       ATGCCACGAACACCGAATCCGCCATCTGTATTGATGACCACCCCTGTCATCGGACCAGAA
60R       ATGCCACGAACACCGAATCCGCCATCTGTATTGATGACCACCCCTGTCATCGGACCAGAA
64R       ATGCCACGAACACCGAATCCGCCATCTGTATTGATGACCACCCCTGTCATCGGACCAGAA
          ************************************************************

57R       TTTTCGCGGGAGGCCAACAGGACATAAGGTCCACAGTAATCGGCCGGAACGGGGGCCATA
60R       TTTTCGCGGGAGGCCAACAGGACATAAGGTCCACAGTAATCGGCCGGAACGGGGGCCATA
64R       TTTTCGCGGGAGGCCAACAGGACATAAGGTCCACAGTAATCGGCCGGAACGGGGGCCATA
          ************************************************************
```

DNA sequences of clones CCA-57, CCA-60, and CCA-64 obtained using primers BBaG00100-F and BBaG00100-R

>57-BBaG00100-F

GGCGTATCACGAGGCAGAATTTCAGATAAAAAAAATCCTTAGCTTTCGCTAAGGATGATTTCTGGAATTCGCGGCCGCTTCTAGATTGACGGC
TAGCTCAGTCCTAGGTACAGTGCTAGCAAAGAGGAGAAAACTAGATGGATACGCAACTTATCATTGATAACGCAGACGTTCCGGCGACTGCTG
CCGCGACCTTTGAACGTCGTAGTCCTACAACCGGCGAATTAGTGACTCGCGCCGCCGCCGCCAGCGTCGCTGACGCAATTGCAGCCGCTGACT
CTGCTGCCGCAGCTTATCGTTCCTGGAGCACTACTGGGCCCACCGAGCGCCGCCGCATCTTGTTGAAAGCCGCCGATTTATTAGAAGCCCGCA
CCCCTGAGTTTTCACGCGTCATGGCCTTGGAAGTGGGTGCATCAGACCTTTGGGCTGGCGTTAACGTAATGCTTGCAGCTAACTTATTTCGCG
AGGCTGCTGCATTAACAACGCAAATTCAGGGGGAGACCATTCCAACAGACAAGGCTGGTGTCTTATCGATGACTGTTCGTCAGCCCGTTGGCG
TGATTTTATCTATTGCCCCGTGGAATGGACCTGTTGTATTAGCTGCTCGTGCCATCGCGTACCCATTGGTCTGCGGAAACACAGTAGTGTTTC
GTGCGTCGGAGTTATCTCCAAAGACGCACATGCTTATCGTGGATGTCTTACGTGACGCTGGCCTGCCGCCAGGAGTCTTGAATGCAGTTACCA
ACGCACCTCAAGATGCACCAGAAGTAGTGGACGCGTTAATCGCACACCCTGCGGTGCGTCGCATTAACTTTACGGGGAGTACCCGTGTGGGGC
GTGTAATTGCAGAAAAAGCAGCTCGTCACTTAAAGCGTTGCCTTCTTGAGCTTGGAGGCAAGGCCCCCCTGGTANTTTTAAATGATGCGGATA
TCGACGAAGCTGTTAAAGCCGCCGTCTTCGGTGCGTTCTTGTACCAAGGGCAGATTTGNNTGTCGACAGAACGTATTGTTGTCGANNAAAAAT
TGCTGATACTTTCGTTGCCCGTTTCGCCGCC

>60F

AGGCGTATNACGAGGCAGAATTTCAGATAAAAAAAATCCTTAGCTTTCGCTAAGGATGATTTCTGGAATTCGCGGCCGCTTCTAGATTTACAG
CTAGCTCAGTCCTAGGTATTATGCTAGCAAAGAGGAGAAAACTAGATGGATACGCAACTTATCATTGATAACGCAGACGTTCCGGCGACTGCT
GCCGCGACCTTTGAACGTCGTAGTCCTACAACCGGCGAATTAGTGACTCGCGCCGCCGCCGCCAGCGTCGCTGACGCAATTGCAGCCGCTGAC
TCTGCTGCCGCAGCTTATCGTTCCTGGAGCACTACTGGGCCCACCGAGCGCCGCCGCATCTTGTTGAAAGCCGCCGATTTATTAGAAGCCCGC
ACCCCTGAGTTTTCACGCGTCATGGCCTTGGAAGTGGGTGCATCAGACCTTTGGGCTGGCGTTAACGTAATGCTTGCAGCTAACTTATTTCGC
GAGGCTGCTGCATTAACAACGCAAATTCAGGGGGAGACCATTCCAACAGACAAGGCTGGTGTCTTATCGATGACTGTTCGTCAGCCCGTTGGC
GTGATTTTATCTATTGCCCCGTGGAATGGACCTGTTGTATTAGCTGCTCGTGCCATCGCGTACCCATTGGTCTGCGGAAACACAGTAGTGTTT
CGTGCGTCGGAGTTATCTCCAAAGACGCACATGCTTATCGTGGATGTCTTACGTGACGCTGGCCTGCCGCCAGGAGTCTTGAATGCAGTTACC
AACGCACCTCAAGATGCACCAGAAGTAGTGGACGCGTTAATCGCACACCCTGCGGTGCGTCGCATTAACTTTACGGGGAGTACCCGTGTGGGG
CGTGTAATTGCAGAAAAAGCAGCTCGTCACTTAAAGCGTTGCCTTCTTGAGCTTGGAGGCAAGGCCCCCCTGGTAGTTTTAGATGATGCGGAT
ATCGACGAAGCTGTTAAAGCCGCCGTCTTCGGTGCGTTCTTGTACCAAGGGCAGATTTGTATGTCGACAGAACGTATTGTTGTCGACGAAAAA
ATTGCTGATACTTTCGTTGCCCGTTTCNCCGCCCGCGCCNNNAATTA

>64F

TCACGAGGCAGAATTTCAGATAAAAAAAATCCTTAGCTTTCGCTAAGGATGATTTCTGGAATTCGCGGCCGCTTCTAGATTTACGGCTAGCTC
AGTCCTAGGTACAATGCTAGCAAAGAGGAGAAAACTAGATGGATACGCAACTTATCATTGATAACGCAGACGTTCCGGCGACTGCTGCCGCGA
CCTTTGAACGTCGTAGTCCTACAACCGGCGAATTAGTGACTCGCGCCGCCGCCGCCAGCGTCGCTGACGCAATTGCAGCCGCTGACTCTGCTG
CCGCAGCTTATCGTTCCTGGAGCACTACTGGGCCCACCGAGCGCCGCCGCATCTTGTTGAAAGCCGCCGATTTATTAGAAGCCCGCACCCCTG
AGTTTTCACGCGTCATGGCCTTGGAAGTGGGTGCATCAGACCTTTGGGCTGGCGTTAACGTAATGCTTGCAGCTAACTTATTTCGCGAGGCTG
CTGCATTAACAACGCAAATTCAGGGGGAGACCATTCCAACAGACAAGGCTGGTGTCTTATCGATGACTGTTCGTCAGCCCGTTGGCGTGATTT
TATCTATTGCCCCGTGGAATGGACCTGTTGTATTAGCTGCTCGTGCCATCGCGTACCCATTGGTCTGCGGAAACACAGTAGTGTTTCGTGCGT
CGGAGTTATCTCCAAAGACGCACATGCTTATCGTGGATGTCTTACGTGACGCTGGCCTGCCGCCAGGAGTCTTGAATGCAGTTACCAACGCAC
CTCAAGATGCACCAGAAGTAGTGGACGCGTTAATCGCACACCCTGCGGTGCGTCGCATTAACTTTACGGGGAGTACCCGTGTGGGGCGTGTAA
TTGCAGAAAAAGCAGCTCGTCACTTAAAGCGTTGCCTTCTTGAGCTTGGAGGCAAGGCCCCCCTGGTAGTTTTAGATGATGCGGATATCGACG
AAGCTGTTAAAGCCGCCGTCTTCGGTGCGTTCTTGTACCAAGGGCAGATTTGTATGTCGACAGAACGTATTGTTGTCGACGAAAAAATTGCTG
ATACTTTCGTTGCCCGTTTCNCCNCCNGCGCCCGCGAATTACCTGNGGGCGATCCTGNNACCTGNNGNNGGN

>57R
ACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCCTGCATAACGCGAAGTAATCTTTTCGGTTTTAAAGAAAAAGGGCAGGGTGGTGACACC
TTGCCCTTTTTTGCCGGACTGCAGCGGCCGCTACTAGTATATAAACGCAGAAAGGCCCACCCGAAGGTGAGCCAGTGTGACTCTAGTAGAGAG
CGTTCACCGACAAACAACAGATAAAACGAAAGGCCCAGTCTTTCGACTGAGCCTTTCGTTTTATTTGATGCCTGGTTATTAATGCAAATCATC
ACCACCACACACCTTCATAATGCCACGAACACCGAATCCGCCATCTGTATTGATGACCACCCCTGTCATCGGACCAGAATTTTCGCGGGAGGC
CAACAGGACATAAGGTCCACAGTAATCGGCCGGAACGGGGGCCATACGCAGGGGAGTATTTTCCACCAGCATAGCCTCCAAATTGTCAACTTG
ATCAAGGGTTTGAGCTGACGTACCAGTTGCCGACAAACCTCCCAAATTTGTGCGCATCCCCCCTGGAGCGACGCCATTTACACGTACTTTTGG
GGCCAACTCGTGAGCCAATTCACGAATCAGTCCCACCAAAGCATGTTTACTTGCAGTGTACAAGGGGCCACCCCCCCCGGGGTAGAAACCTGC
ATTAGAAACGGTAAAGATTAACGAACCCTGGGATTTTACCAGTTCAGCTAACGCTGCTTTGGCCCCAAGCAGTGCAGCTTTTACGTTCACTGC
GAATAATTCGTCGAATGCGCGTGAGATGCTCCCGGCGTCCATCTGGGGCAATGTCTGGAAAAAATCGAAGACTCCGGCGTTTCCGACAAAGTT
GTCAAGGCGACCGAAACGTGCAACTGTTTTCTGGACCACAAGAACGTTATCTTCATACAACGTCACGTCTCCTACCACTACTTCTACTGCGTC
CCCAAATTCACGAGCAAGCTCACGGGCGCGTTCGCCAGAGCGTTCAAGGACCCCCACACGTCCACCCTCATTGATGAAACGTTCCACTAATGC
TTTGCCCAGTCCA

>60R
CGACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCCTGCATAACGCGAAGTAATCTTTTCGGTTTTAAAGAAAAAGGGCAGGGTGGTGACA
CCTTGCCCTTTTTTGCCGGACTGCAGCGGCCGCTACTAGTATATAAACGCAGAAAGGCCCACCCGAAGGTGAGCCAGTGTGACTCTAGTAGAG
AGCGTTCACCGACAAACAACAGATAAAACGAAAGGCCCAGTCTTTCGACTGAGCCTTTCGTTTTATTTGATGCCTGGTTATTAATGCAAATCA
TCACCACCACACACCTTCATAATGCCACGAACACCGAATCCGCCATCTGTATTGATGACCACCCCTGTCATCGGACCAGAATTTTCGCGGGAG
GCCAACAGGACATAAGGTCCACAGTAATCGGCCGGAACGGGGGCCATACGCAGGGGAGTATTTTCCACCAGCATAGCCTCCAAATTGTCAACT
TGATCAAGGGTTTGAGCTGACGTACCAGTTGCCGACAAACCTCCCAAATTTGTGCGCATCCCCCCTGGAGCGACGCCATTTACACGTACTTTT
GGGGCCAACTCGTGAGCCAATTCACGAATCAGTCCCACCAAAGCATGTTTACTTGCAGTGTACAAGGGGCCACCCCCCCCGGGGTAGAAACCT
GCATTAGAAACGGTAAAGATTAACGAACCCTGGGATTTTACCAGTTCAGCTAACGCTGCTTTGGCCCCAAGCAGTGCAGCTTTTACGTTCACT
GCGAATAATTCGTCGAATGCGCGTGAGATGCTCCCGGCGTCCATCTGGGGCAATGTCTGGAAAAAATCGAAGACTCCGGCGTTTCCGACAAAG
TTGTCAAGGCGACCGAAACGTGCAACTGTTTTCTGGACCACAAGAACGTTATCTTCATACAACGTCACGTCTCCTACCACTACTTCTACTGCG
TCCCCAAATTCACGAGCAAGCTCACGGGCGCGTTCGCCAGAGCGTTCAAGGACCCCCACACGTCCACCCTCATTGATGAAACGTTCCACTAAT
GCTTTGCCCAGTCCANAGCCNCCACCCGGNATGATTGGCACTGGATTATTCAACCAGC

>64R
GACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCCTGCATAACGCGAAGTAATCTTTTCGGTTTTAAAGAAAAAGGGCAGGGTGGTGACACCTT
GCCCTTTTTTGCCGGACTGCAGCGGCCGCTACTAGTATATAAACGCAGAAAGGCCCACCCGAAGGTGAGCCAGTGTGACTCTAGTAGAGAGCG
TTCACCGACAAACAACAGATAAAACGAAAGGCCCAGTCTTTCGACTGAGCCTTTCGTTTTATTTGATGCCTGGTTATTAATGCAAATCATCAC
CACCACACACCTTCATAATGCCACGAACACCGAATCCGCCATCTGTATTGATGACCACCCCTGTCATCGGACCAGAATTTTCGCGGGAGGCCA
ACAGGACATAAGGTCCACAGTAATCGGCCGGAACGGGGGCCATACGCAGGGGAGTATTTTCCACCAGCATAGCCTCCAAATTGTCAACTTGAT
CAAGGGTTTGAGCTGACGTACCAGTTGCCGACAAACCTCCCAAATTTGTGCGCATCCCCCCTGGAGCGACGCCATTTACACGTACTTTTGGGG
CCAACTCGTGAGCCAATTCACGAATCAGTCCCACCAAAGCATGTTTACTTGCAGTGTACAAGGGGCCACCCCCCCCGGGGTAGAAACCTGCAT
TAGAAACGGTAAAGATTAACGAACCCTGGGATTTTACCAGTTCAGCTAACGCTGCTTTGGCCCCAAGCAGTGCAGCTTTTACGTTCACTGCGA
ATAATTCGTCGAATGCGCGTGAGATGCTCCCGGCGTCCATCTGGGGCAATGTCTGGAAAAAATCGAAGACTCCGGCGTTTCCGACAAAGTTGT
CAAGGCGACCGAAACGTGCAACTGTTTTCTGGACCACAAGAACGTTATCTTCATACAACGTCACGTCTCCTACCACTACTTCTACTGCGTCCC
CAAATTCACGAGCAAGCTCACGGGCGCGTTCGCCAGAGCGTTCAAGGACCCCCACACGTCCACCCTCATTGATGAAACGTTCCACTAATGGT

DNA sequence of clone CCA-57-Forward blast

57Fdehydrogenase [Burkholderia sp. Bk]
Sequence ID: WP_088176951.1Length: 483Number of Matches: 1

Alignment statistics for match #1

Score	Expect	Method	Identities	Positives	Gaps
572 bits(1474)	0.0	Compositional matrix adjust.	289/296(98%)	291/296(98%)	0/296

```
Query  1    MDTQLIIDNADVPATAAATFERRSPTTGELVTRAAAASVADAIAAADSAAAAYRSWSTTG  60
            MDTQLIIDNADVPATAAATFERRSPTTGELVTRAAAASVADAIAAADSAAAAYRSWSTTG
Sbjct  1    MDTQLIIDNADVPATAAATFERRSPTTGELVTRAAAASVADAIAAADSAAAAYRSWSTTG  60
```

```
Query  61   PTERRRILLKAADLLEARTPEFSRVMALEVGASDLWAGVNVMLAANLFREAAALTTQIQG  120
            PTERRRILLKAADLLEARTPEFSRVMALEVGASDLWAGVNVMLAANLFREAAALTTQIQG
Sbjct  61   PTERRRILLKAADLLEARTPEFSRVMALEVGASDLWAGVNVMLAANLFREAAALTTQIQG  120

Query  121  ETIPTDKAGVLSMTVRQPVGVILSIAPWNGPVVLAARAIAYPLVCGNTVVFRASELSPKT  180
            ETIPTDKAGVLSMTVRQPVGVILSIAPWNGPVVLAARAIAYPLVCGNTVVFRASELSPKT
Sbjct  121  ETIPTDKAGVLSMTVRQPVGVILSIAPWNGPVVLAARAIAYPLVCGNTVVFRASELSPKT  180

Query  181  HMLIVDVLRDAGLPPGVLNAVTNAPQDAPEVVDALIAHPAVRRINFTGSTRVGRVIAEKA  240
            HMLIVDVLRDAGLPPGVLNAVTNAPQDAPEVVDALIAHPAVRRINFTGSTRVGRVIAEKA
Sbjct  181  HMLIVDVLRDAGLPPGVLNAVTNAPQDAPEVVDALIAHPAVRRINFTGSTRVGRVIAEKA  240

Query  241  ARHLKRCLLELGGKAPLVXLNDADIDEAVKAAVFGAFLYQGQIXXSTERIVVXXKL  296
            ARHLKRCLLELGGKAPLV L+DADIDEAVKAAVFGAFLYQGQI  STERIVV  K+
Sbjct  241  ARHLKRCLLELGGKAPLVVLDDADIDEAVKAAVFGAFLYQGQICMSTERIVVDEKI  296
```

DNA sequence of clone CCA-57-Forward Reverse

3-(cis-5,6-dihydroxycyclohexa-1,3-dien-1-yl)propanoate dehydrogenase [Burkholderia sp. Bk]
Sequence ID: WP_088176957.1Length: 272Number of Matches: 1

Alignment statistics for match #1

Score	Expect	Method	Identities	Positives	Gaps
511 bits(1316)	0.0	Compositional matrix adjust.	256/256(100%)	256/256(100%)	0/256

```
Query  1    GLGKALVERFINEGGRVGVLERSGERARELAREFGDAVEVVVGDVTLYEDNVLVVQKTVA  60
            GLGKALVERFINEGGRVGVLERSGERARELAREFGDAVEVVVGDVTLYEDNVLVVQKTVA
Sbjct  17   GLGKALVERFINEGGRVGVLERSGERARELAREFGDAVEVVVGDVTLYEDNVLVVQKTVA  76

Query  61   RFGRLDNFVGNAGVFDFFQTLPQMDAGSISRAFDELFAVNVKAALLGAKAALAELVKSQG  120
            RFGRLDNFVGNAGVFDFFQTLPQMDAGSISRAFDELFAVNVKAALLGAKAALAELVKSQG
Sbjct  77   RFGRLDNFVGNAGVFDFFQTLPQMDAGSISRAFDELFAVNVKAALLGAKAALAELVKSQG  136

Query  121  SLIFTVSNAGFYPGGGGPLYTASKHALVGLIRELAHELAPKVRVNGVAPGGMRTNLGGLS  180
            SLIFTVSNAGFYPGGGGPLYTASKHALVGLIRELAHELAPKVRVNGVAPGGMRTNLGGLS
Sbjct  137  SLIFTVSNAGFYPGGGGPLYTASKHALVGLIRELAHELAPKVRVNGVAPGGMRTNLGGLS  196

Query  181  ATGTSAQTLDQVDNLEAMLVENTPLRMAPVPADYCGPYVLLASRENSGPMTGVVINTDGG  240
            ATGTSAQTLDQVDNLEAMLVENTPLRMAPVPADYCGPYVLLASRENSGPMTGVVINTDGG
Sbjct  197  ATGTSAQTLDQVDNLEAMLVENTPLRMAPVPADYCGPYVLLASRENSGPMTGVVINTDGG  256

Query  241  FGVRGIMKVCGGDDLH  256
            FGVRGIMKVCGGDDLH
Sbjct  257  FGVRGIMKVCGGDDLH  272
```

Translation

>57F

MDTQLIIDNADVPAT
AAATFERRSPTTGELVTRAAAASVADAIAAADSAAAAYRSWSTTGPTERRRILLKAADLL
EARTPEFSRVMALEVGASDLWAGVNVMLAANLFREAAALTTQIQGETIPTDKAGVLSMTV
RQPVGVILSIAPWNGPVVLAARAIAYPLVCGNTVVFRASELSPKTHMLIVDVLRDAGLPP
GVLNAVTNAPQDAPEVVDALIAHPAVRRINFTGSTRVGRVIAEKAARHLKRCLLELGGKA
PLVXLNDADIDEAVKAAVFGAFLYQGQIXXSTERIVVXXKLLILSLPVSP

>57R

GLGKALVERFINEGGRVGVLERSGERARELAREFGDAVEVVVGDVTLYEDNVLVVQKTVA
RFGRLDNFVGNAGVFDFFQTLPQMDAGSISRAFDELFAVNVKAALLGAKAALAELVKSQG
SLIFTVSNAGFYPGGGGPLYTASKHALVGLIRELAHELAPKVRVNGVAPGGMRTNLGGLS
ATGTSAQTLDQVDNLEAMLVENTPLRMAPVPADYCGPYVLLASRENSGPMTGVVINTDGG
FGVRGIMKVCGGDDLH

Sequence request

Primers to be synthetized by Retrogen

BBa_G00100_F	5'-tgccacctgacgtctaagaa-3"
BBa_G00101_R	5'- attaccgcctttgagtgagc-3"

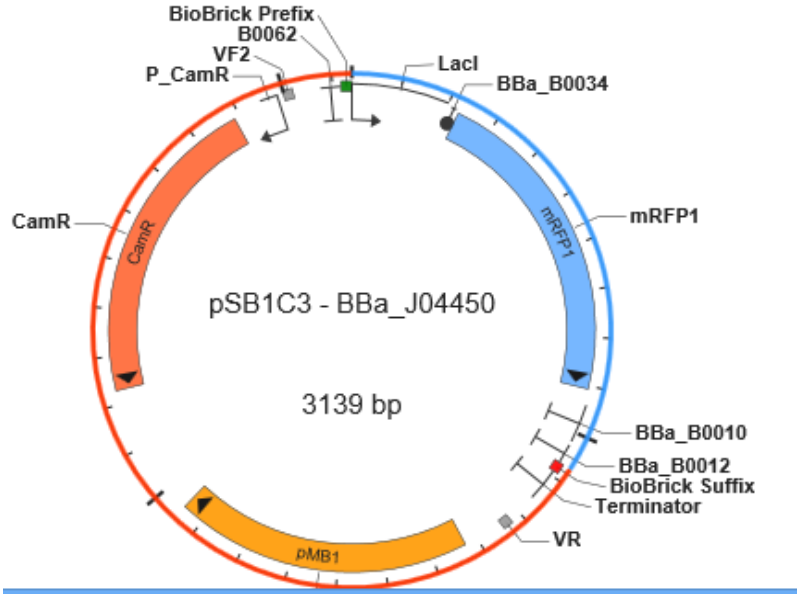
Sample Number	Sample Description	Primer
48	DNA miniprep	BBa_G00100_F
49	DNA miniprep	BBa_G00100_F
51	DNA miniprep	BBa_G00100_F
53	DNA miniprep	BBa_G00100_F
54	DNA miniprep	BBa_G00100_F
55	DNA miniprep	BBa_G00100_F
57	DNA miniprep	BBa_G00100_F
59	DNA miniprep	BBa_G00100_F
60	DNA miniprep	BBa_G00100_F
61	DNA miniprep	BBa_G00100_F
64	DNA miniprep	BBa_G00100_F
65	DNA miniprep	BBa_G00100_F
48	DNA miniprep	BBa_G00101_R
49	DNA miniprep	BBa_G00101_R
51	DNA miniprep	BBa_G00101_R
53	DNA miniprep	BBa_G00101_R
54	DNA miniprep	BBa_G00101_R
55	DNA miniprep	BBa_G00101_R
57	DNA miniprep	BBa_G00101_R
59	DNA miniprep	BBa_G00101_R
60	DNA miniprep	BBa_G00101_R
61	DNA miniprep	BBa_G00101_R
64	DNA miniprep	BBa_G00101_R
65	DNA miniprep	BBa_G00101_R

SeqId	Sample	Primer	Date	Phred Q20	Comments

40776-59	48	BBaG00100-F	Aug 7 2017	phd qual 1133 fasta	Great Sequence
40776-60	48	BBaG00100-R	Aug 7 2017	phd qual 629 fasta	black epi, use 18723-BBa-R 40776-60|D8 REDO GC
40776-61	49	BBaG00100-F	Aug 7 2017	phd qual 1122 fasta	Great Sequence
40776-62	49	BBaG00100-R	Aug 7 2017	phd qual 675 fasta	black epi, use 18723-BBa-R 40776-62|F8 REDO GC
40776-63	51	BBaG00100-F	Aug 7 2017	phd qual 1102 fasta	Great Sequence
40776-64	51	BBaG00100-R	Aug 7 2017	phd qual 653 fasta	black epi, use 18723-BBa-R 40776-64|H8 REDO GC
40776-65	53	BBaG00100-F	Aug 7 2017	phd qual 1121 fasta	Great Sequence
40776-66	53	BBaG00100-R	Aug 7 2017	phd qual 480 fasta	black epi, use 18723-BBa-R 40776-66|B9 REDO GC
40776-67	54	BBaG00100-F	Aug 7 2017	phd qual 1060 fasta	Great Sequence
40776-68	54	BBaG00100-R	Aug 7 2017	phd qual 663 fasta	black epi, use 18723-BBa-R 40776-68|D9 REDO GC
40776-69	55	BBaG00100-F	Aug 7 2017	phd qual 1055 fasta	Great Sequence
40776-70	55	BBaG00100-R	Aug 7 2017	phd qual 482 fasta	black epi, use 18723-BBa-R 40776-70|F9 REDO GC
40776-71	57	BBaG00100-F	Aug 7 2017	phd qual 1086 fasta	Great Sequence
40776-72	57	BBaG00100-R	Aug 7 2017	phd qual 1112 fasta	black epi, use 18723-BBa-R 40776-72|H9 REDO GC
40776-73	59	BBaG00100-F	Aug 7 2017	phd qual 1111 fasta	Great Sequence
40776-74	59	BBaG00100-R	Aug 7 2017	phd qual 1090 fasta	black epi, use 18723-BBa-R 40776-74|B10 REDO GC
40776-75	60	BBaG00100-F	Aug 7 2017	phd qual 1115 fasta	Great Sequence
40776-76	60	BBaG00100-R	Aug 7 2017	phd qual 1103 fasta	black epi, use 18723-BBa-R 40776-76|D10 REDO GC
40776-77	61	BBaG00100-F	Aug 7 2017	phd qual 1084 fasta	Great Sequence
40776-78	61	BBaG00100-R	Aug 7 2017	phd qual 1099 fasta	black epi, use 18723-BBa-R 40776-78|F10 REDO GC
40776-79	64	BBaG00100-F	Aug 7 2017	phd qual 1122 fasta	Great Sequence
40776-80	64	BBaG00100-R	Aug 7 2017	phd qual 1072 fasta	black epi, use 18723-BBa-R 40776-80|H10 REDO GC
40776-81	65	BBaG00100-F	Aug 7 2017	phd qual 1110 fasta	Great Sequence
40776-82	65	BBaG00100-R	Aug 7 2017	phd qual 1095 fasta	black epi, use 18723-BBa-R 40776-82|B11 REDO GC

There are 24 samples.

Plasmid map



Sequence verification

Fluorene clones for deposit in registry

CCA-66, CCA-68, and CCA-70.

Sequence Alignment of clones CCA-66, CCA-68, and CCA-70.

Results:

- All 3 clones are matching the template sequence of the first gene of the fluorene pathway as shown in the alignment below performed using clustal omega. The difference between the 3 clones is the promoter (strong, weak, and moderate) but the ORF is the same (flnB).
- The forward primer BBaG00100-F worked well. The reverse primer BBaG00100-R did not work (as reported on IGEM for some sequences).
- The translation matches the flnB amino acid sequence.

Clustal Alignment

```
CLUSTAL O(1.2.4) multiple sequence alignment


68      ---AGGCGTATCACGAGGCAGAATTTCAGATAAAAAAAATCCTTAGCTTTCGCTAAGGAT
66      --TAGGCGTATCACGAGGCAGAATTTCAGATAAAAAAAATCCTTAGCTTTCGCTAAGGAN
70      AATAGGCGTATCACGAGGCAGAATTTCAGATAAAAAAAATCCTTAGCTTTCGCTAAGGAT
           ***************************************************.

68      GATTTCTGGAATTCGCGGCCGCTTCTAGATTTACAGCTAGCTCAGTCCTAGGTATTATGC
```

```
66      GATTTCTGGAATTCGCGGCCGCTTCTAGATTGACGGCTAGCTCAGTCCTAGGTACAGTGC
70      GATTTCTGGAATTCGCGGCCGCTTCTAGATTTACGGCTAGCTCAGTCCTAGGTACAATGC
        ****************************** **.****************** :.***

68      TAGCAAAGAGGAGAAAACTAGATGTCCGAATCAGGGGGTGGGACTGTTGCTACCGCACGT
66      TAGCAAAGAGGAGAAAACTAGATGTCCGAATCAGGGGGTGGGACTGTTGCTACCGCACGT
70      TAGCAAAGAGGAGAAAACTAGATGTCCGAATCAGGGGGTGGGACTGTTGCTACCGCACGT
        ************************************************************

68      CAGCGCCAGTTGGTGGAACGTGCATTGGGCGAGTGGCAAGGAGAAGTGGCGGGTCGCGTA
66      CAGCGCCAGTTGGTGGAACGTGCATTGGGCGAGTGGCAAGGAGAAGTGGCGGGTCGCGTA
70      CAGCGCCAGTTGGTGGAACGTGCATTGGGCGAGTGGCAAGGAGAAGTGGCGGGTCGCGTA
        ************************************************************

68      ATTGTGGTAACAGGTGGGGCTCGCGGGATCGGTCGCAGTTTATGTGAAGGTCTTTTACGC
66      ATTGTGGTAACAGGTGGGGCTCGCGGGATCGGTCGCAGTTTATGTGAAGGTCTTTTACGC
70      ATTGTGGTAACAGGTGGGGCTCGCGGGATCGGTCGCAGTTTATGTGAAGGTCTTTTACGC
        ************************************************************

68      GCAGGTGCCAAGGTCGTGGCCGCTGATTTAACCTGGGACGACGCCGATGACTTCCGCAAA
66      GCAGGTGCCAAGGTCGTGGCCGCTGATTTAACCTGGGACGACGCCGATGACTTCCGCAAA
70      GCAGGTGCCAAGGTCGTGGCCGCTGATTTAACCTGGGACGACGCCGATGACTTCCGCAAA
        ************************************************************

68      CAATTAGAGTCCGACGGCTCTGGTATGGCCGTAGATATGGATATTACAGACGATGATGCC
66      CAATTAGAGTCCGACGGCTCTGGTATGGCCGTAGATATGGATATTACAGACGATGATGCC
70      CAATTAGAGTCCGACGGCTCTGGTATGGCCGTAGATATGGATATTACAGACGATGATGCC
        ************************************************************

68      TTGGACGCTGCCCGTGACGCAGTAATCGACCGCTTCGGAACCGTTGATGTCTTGGTGAAT
66      TTGGACGCTGCCCGTGACGCAGTAATCGACCGCTTCGGAACCGTTGATGTCTTGGTGAAT
70      TTGGACGCTGCCCGTGACGCAGTAATCGACCGCTTCGGAACCGTTGATGTCTTGGTGAAT
        ************************************************************

68      AACGCTTCGCTGGTCTCTGAGACTTTGTTTCCACCAACGGGGCACCGTAATACCCTGGAC
66      AACGCTTCGCTGGTCTCTGAGACTTTGTTTCCACCAACGGGGCACCGTAATACCCTGGAC
70      AACGCTTCGCTGGTCTCTGAGACTTTGTTTCCACCAACGGGGCACCGTAATACCCTGGAC
        ************************************************************

68      ACGACAGATCGCGACTGGGAGGTAATGTTTGGTGTGAATGTCTTTGGAACACTTAAGGCG
66      ACGACAGATCGCGACTGGGAGGTAATGTTTGGTGTGAATGTCTTTGGAACACTTAAGGCG
70      ACGACAGATCGCGACTGGGAGGTAATGTTTGGTGTGAATGTCTTTGGAACACTTAAGGCG
        ************************************************************
```

```
>Translation of 70
MSESGGGTVATAR
QRQLVERALGEWQGEVAGRVIVVTGGARGIGRSLCEGLLRAGAKVVAADLTWDDADDFRK
QLESDGSGMAVDMDITDDDALDAARDAVIDRFGTVDVLVNNASLVSETLFPPTGHRNTLD
TTDRDWEVMFGVNVFGTLKAIRRFIEPMRAQQRGSIVNVVSSGVLAVAAGGGYHGLRPWT
VEMPYQATKAAVMALTFYLAEEVRGDGVAVNAIMPGHTRASWFDATARAFNEQGIAYFMR
PAIPEHLLPISLFLAAQESAGASGRLYYVPXXNYDH
```

```
>66_F
TAGGCGTATCACGAGGCAGAATTTCAGATAAAAAAAATCCTTAGCTTTCGCTAAGGANGATTTCTGGAATTCGCGGCCGCTTCTAGATTGACGGCTAGCTCAGTCCTAGGTA
CAGTGCTAGCAAAGAGGAGAAAACTAGATGTCCGAATCAGGGGGTGGGACTGTTGCTACCGCACGTCAGCGCCAGTTGGTGGAACGTGCATTGGGCGAGTGGCAAGGAG
AAGTGGCGGGTCGCGTAATTGTGGTAACAGGTGGGGCTCGCGGGATCGGTCGCAGTTTATGTGAAGGTCTTTTACGCGCAGGTGCCAAGGTCGTGGCCGCTGATTTAACCT
GGGACGACGCCGATGACTTCCGCAAACAATTAGAGTCCGACGGCTCTGGTATGGCCGTAGATATGGATATTACAGACGATGATGCCTTGGACGCTGCCCGTGACGCAGTAA
```

TCGACCGCTTCGGAACCGTTGATGTCTTGGTGAATAACGCTTCGCTGGTCTCTGAGACTTTGTTTCCACCAACGGGGCACCGTAATACCCTGGACACGACAGATCGCGACTGG
GAGGTAATGTTTGGTGTGAATGTCTTTGGAACACTTAAGGCGATTCGTCGCTTCATCGAGCCAATGCGCGCTCAACAGCGCGGGNTCGATTGTCGACNN

>68_F

AGGCGTATCACGAGGCAGAATTTCAGATAAAAAAAATCCTTAGCTTTCGCTAAGGATGATTTCTGGAATTCGCGGCCGCTTCTAGATTTACAGCTAGCTCAGTCCTAGGTATT
ATGCTAGCAAAGAGGAGAAAACTAGATGTCCGAATCAGGGGGTGGGACTGTTGCTACCGCACGTCAGCGCCAGTTGGTGGAACGTGCATTGGGCGAGTGGCAAGGAGAA
GTGGCGGGTCGCGTAATTGTGGTAACAGGTGGGGCTCGCGGGATCGGTCGCAGTTTATGTGAAGGTCTTTTACGCGCAGGTGCCAAGGTCGTGGCCGCTGATTTAACCTGG
GACGACGCCGATGACTTCCGCAAACAATTAGAGTCCGACGGCTCTGGTATGGCCGTAGATATGGATATTACAGACGATGATGCCTTGGACGCTGCCCGTGACGCAGTAATC
GACCGCTTCGGAACCGTTGATGTCTTGGTGAATAACGCTTCGCTGGTCTCTGAGACTTTGTTTCCACCAACGGGGCACCGTAATACCCTGGACACGACAGATCGCGACTGGG
AGGTAATGTTTGGTGTGAATGTCTTTGGAACACTTAAGGCGATTCGTCGCTTCATCGAGCCAATGCGCGCTCAACAGCGCGGTTCGATTGTCAACGTGGTAAGCAGTGGCGT
CCTTGCAGTCGCAGCTGGCGGGGGATACCATGGCTTGCGTCCATGGACCGTTGAGATGCCCTATCAGGCTACTAAAGCAGCTGTCATGGCTCTTACATTCTACTTGGCCGAA
AAGGTGCGCGGCGATGGGGTGGCGGTCAATGCTATCATGCCTGGTCACACCCGCGCTTNTNGGTTTG

>70_F

AATAGGCGTATCACGAGGCAGAATTTCAGATAAAAAAAATCCTTAGCTTTCGCTAAGGATGATTTCTGGAATTCGCGGCCGCTTCTAGATTTACGGCTAGCTCAGTCCTAGGT
ACAATGCTAGCAAAGAGGAGAAAACTAGATGTCCGAATCAGGGGGTGGGACTGTTGCTACCGCACGTCAGCGCCAGTTGGTGGAACGTGCATTGGGCGAGTGGCAAGGA
GAAGTGGCGGGTCGCGTAATTGTGGTAACAGGTGGGGCTCGCGGGATCGGTCGCAGTTTATGTGAAGGTCTTTTACGCGCAGGTGCCAAGGTCGTGGCCGCTGATTTAACC
TGGGACGACGCCGATGACTTCCGCAAACAATTAGAGTCCGACGGCTCTGGTATGGCCGTAGATATGGATATTACAGACGATGATGCCTTGGACGCTGCCCGTGACGCAGTA
ATCGACCGCTTCGGAACCGTTGATGTCTTGGTGAATAACGCTTCGCTGGTCTCTGAGACTTTGTTTCCACCAACGGGGCACCGTAATACCCTGGACACGACAGATCGCGACTG
GGAGGTAATGTTTGGTGTGAATGTCTTTGGAACACTTAAGGCGATTCGTCGCTTCATCGAGCCAATGCGCGCTCAACAGCGCGGTTCGATTGTCAACGTGGTAAGCAGTGGC
GTCCTTGCAGTCGCAGCTGGCGGGGGATACCATGGCTTGCGTCCATGGACCGTTGAGATGCCCTATCAGGCTACTAAAGCAGCTGTCATGGCTCTTACATTCTACTTGGCCGA
AGAGGTGCGCGGCGATGGGGTGGCGGTCAATGCTATCATGCCTGGTCACACCCGCGCTTCTTGGTTTGATGCGACCGCTCGTGCCTTTAATGAGCAGGGGATCGCATACTTC
ATGCGCCCTGCTATTCCCGAGCACTTGCTTCCTATCTCCTTGTTCCTTGCAGCGCAGGAATCCGCTGGCGCCTCTGGGCGTCTTTACTATGTGCCCGANNGGAACTACGACCAC