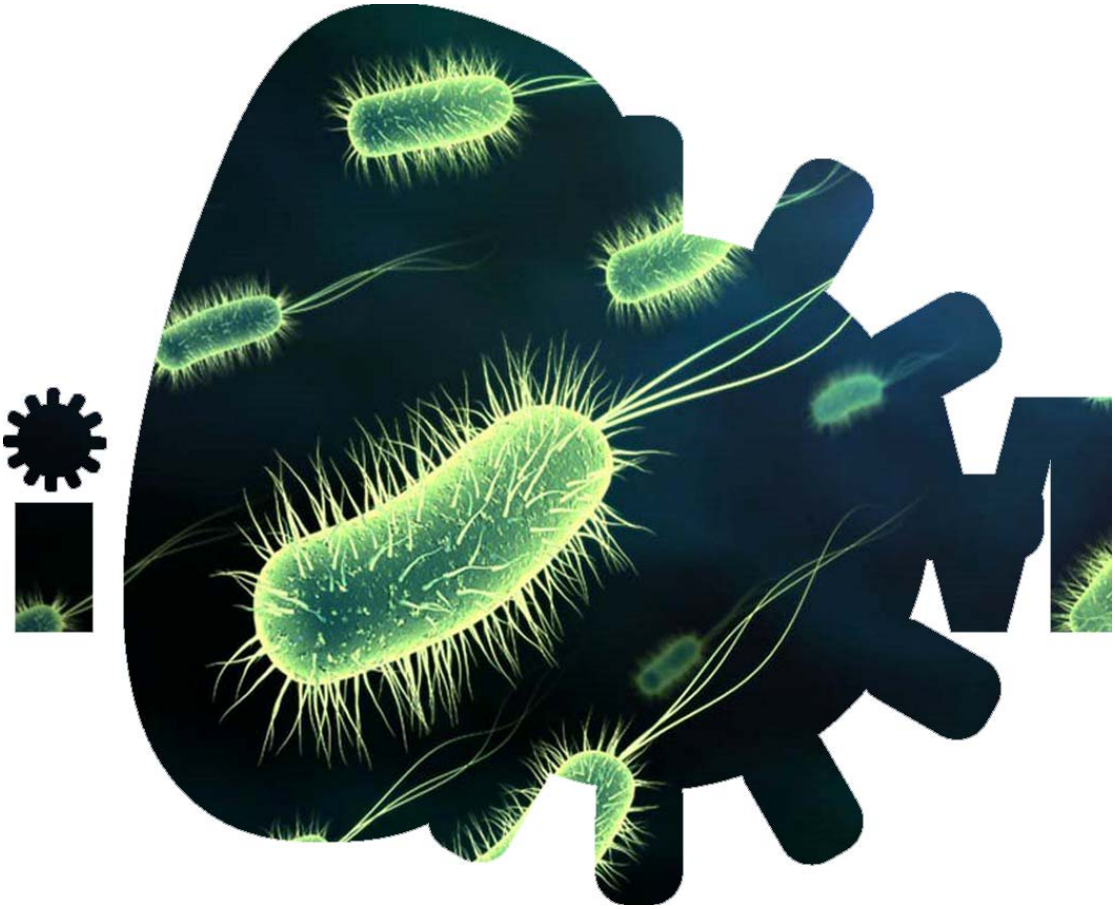

iGEM TU Delft 2010

Research Proposal

Delft, The Netherlands
Version 1 - June 2010
http://2010.igem.org/Team:TU_Delft

TU Delft
Technische Universiteit Delft

iGEM

INTRODUCTION

The International Genetically Engineered Machine (iGEM) competition is an annual synthetic biology competition for undergraduate students from all over the world. Student teams are encouraged to work on a project of their own choice over the summer and to present their results at the Massachusetts Institute of Technology (MIT) in Boston (USA) during the iGEM Jamboree in November.

The general goal of iGEM is to build "simple" biological systems from standard, interchangeable parts (Biobricks) and operate them in living cells. Each team receives a compilation of the best biobricks at the beginning of the project to use, adapt and incorporate in their own project and each team is required to create a number of biobricks themselves. These biobricks are all submitted into the Registry of Standard Biological Parts (http://partsregistry.org), a continuously growing collection of genetic parts that can be mixed and matched to build synthetic biology devices and systems. The Registry provides a resource of available genetic parts to iGEM teams and is based on the principle of "get some, give some". Registry users can use the parts and information available from the Registry in designing their engineered biological systems. In exchange, the expectation is that Registry users will, in turn, contribute back information and data on existing parts and new parts that they make to grow and improve this community resource.

Since 2004, undergraduate teams from all over the world have worked on the most diverse projects that intertwine the principles of Biology and Engineering. The competition not only determines the worth of projects solely based on biological merit, but also requires teams to examine their project as a whole. This includes aspects such as modelling of genetic circuits, ethical and safety aspects, marketing the project and educating the public about iGEM and Synthetic Biology.

The past two years TU Delft teams have also participated in this ever-growing competition, and each year have booked good success. Now, a new year brings a new team with fresh idea's and a whole new project. This booklet was designed to provide information on the project, in particular how we propose to achieve our goals over the summer to create another award-winning iGEM project from the TU Delft.

The Delft University of Technology
international Genetically Engineered Machine (iGEM)
Team 2010

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FACTSHEET

Team name	TU Delft iGEM Team 2010
Number of student members	10
Research Period	June - November 2010
Location	Biotechnology (Kluyver laboratory) Julianalaan 67, 2628 BC Delft, The Netherlands
Office Room Number	0.640
Office Phone Number	015-2781625
Laboratory Room Numbers	Main lab: 0.710 0.670, 0.671, HTS Lab, 2.080 (GC)
Finale	MIT, Boston, USA (November 2010)

PARTICIPATING STUDENTS

Student	Background	E-mail	Phone
Luke Bergwerff	MSc. Biochemical Eng.	deviouslyminded@hotmail.com	06-18098333
Pieter van Boheemen	MSc. Functional Genomics	pietervanboheemen@gmail.com	06-14153298
Nadine Bongaerts	BSc. LS&T	nbongaerts@hotmail.com	06-22879283
Eva Brinkman	MSc. Functional Genomics	ekbrinkman@hotmail.com	06-55938733
Jelmer Cnossen	MSc. Bioinformatics	j.cnossen@gmail.com	06-48709790
Hugo Cueto-Rojas	MSc. Biochemical Eng.	h.f.cuetorojas@gmail.com	06-26525090
Kira Schipper	MSc. Biochemical Eng.	kiraschipper@gmail.com	06-40055467
Mia Urem	MSc. Functional Genomics	dragon_star_17@msn.com	06-40374462
Ramon van der Valk	MSc. Molecular Genetics	hannibaru@hotmail.com	06-18904889
Mathias Voges	MSc. Cell Factory	mjvoges@gmail.com	06-28965676

ADVISORS/SUPERVISORS

Name	Function	E-mail	Phone
Aljoscha Wahl	General supervisor	s.a.wahl@tudelft.nl	015 2783193
Esengül Yildirim	Lab supervisor	E.Yildirim@tudelft.nl	015 2788475
Alessandro Abate	Advisor	a.abate@tudelft.nl	015 2785606
Dominico Bellomo	Advisor	domenico.bellomo@gmail.com	015 2785859
Stefan de Kok	Advisor	S.deKok@tudelft.nl	015 2787466
Amit Deshmukh	Advisor	atdeshmukh@gmail.com	015 2785307

RESEARCH – THE PROJECT

Hydrocarbons (n-alkanes, cyclic hydrocarbons) are environmentally toxic compounds. The 2010 iGEM team will focus on the degradation hydrocarbons in aqueous environments. The degradation in aqueous environments, with high salt concentration, faces several challenges that will be addressed in five subprojects: (1) alkane degradation, (2) salt tolerance, (3) emulsifier production, (4) hydrocarbon sensing and (5) solvent tolerance. Each sub-part is to be executed by a sub-group of students.Our aim will be to design BioBricks to facilitate each process and to then characterize them in *E.coli* K12.

The respective genes are first introduced using RBS expression vectors. Because the five available RBS promotors are not characterized, initially a reporter study using GFP will be performed. Through this characterization the whole team can experience working with BioBricks prior to working on the main project.

1. IGEM STANDARDS

iGEM headquarters supplies each participating team with a number of essential components for the formation of biobricks, namely:

- Existing BioBricks: (3) 384 well plates containing dried DNA of a selection of BioBricks (plasmids) from the registry
- Linearized Plasmid Backbones for pSB1C3, pSB1T3, and pSB1A3

1.1. PLASMIDS & ANTIBIOTICS

The biobricks are supplied as an insert in a plasmid with a certain antibiotic resistance, which is denoted by a letter in the plasmid name[1] (Ampicillin (A), chloramphenicol (C), erythromycin (E), gentamycin (G), kanamycin (K), neomycin (N), nalidixic acid (Na), rifampicin (R), spectinomycin (S), streptomycin (St), tetracycline (T), trimethoprim (Tm) and zeocin (Z)).

We plan to use 6 plasmids from the BioBrick collection, 4 antibiotic resistances are used (table 1).

TABLE 1 – PLASMIDS USED FOR IGEM BIOBRICK FORMATION

Plasmid	Selection marker	Insert	Location	Copy number
pSB1A3	Ampicillin	mRFP1	Plate 1 well 1C	High
pSB1A3-linearized	Ampicillin	-	-	High
pSB1K3	Kanamycin	mRFP1	Plate 1 well 5A	High
pSB1T3	Tetracycline	mRFP1	Plate 1 well 7A	High
pSB1T3-linearized	Tetracycline	-	-	High
pSB1C3	Chloraphenicol	mRFP1	Plate 1 well 3A	High
pSB1C3-linearized	Chloraphenicol	-	-	High
pSB3T5	Tetracycline	mRFP1	Plate 1 well 7C	Medium
pSB3C5	Chloraphenicol	mRFP1	Plate 1 well 3C	Medium

Thus, the antibiotics we will be using are:

- Ampicillin
- Chloramphenicol
- Kanamycin
- Tetracycline

[1] http://partsregistry.org/wiki/index.php/Help:Plasmids/Nomenclature

1.2. EXISTING BIOBRICKS

We will be using a number of existing BioBricks, and for now that list contains only various RBSs and a promoter family, as well as GFP and RFP genes. These can be found in table 2. More information on these BioBricks can be found on the website of the standard registry of parts (http://partsregistry.org).

TABLE 2 – EXISTING BIOBRICKS TO BE USED

BioBrick	Feature	Plasmid	Part Type	Location
BBa_J61100	RBS Anderson family	pSB1A2	RBS	5J
BBa_J61101	RBS Anderson family	pSB1A2	RBS	5L
BBa_J61107	RBS Anderson family	pSB1A2	RBS	5N
BBa_J61117	RBS Anderson family	pSB1A2	RBS	11L
BBa_J61127	RBS Anderson family	pSB1A2	RBS	11N
BBa_J23100-119	Constitutive promoter library	J61002[2]	Promoter	Multiple
BBa_E0040	GFP	pSB1A2	Coding GFP	15K
BBa_E0240	GFP Generator	pSB1A2	RET[3]	12M
BBa_E0422	YFP Generator	pSB1A2	RET	8M
BBa_B0032	Medium strength	pSB1A2	RBS	2I
BBa_B0015	Double forward	pSB1AK2	Terminator	23L
BBa_J04450	RFP	[4]	Reporter	Multiple
BBa_J23109	Medium strength	pSB1A2	Promoter	2G
BBa_I13401	GFP-terminator	pSB1A2	Reporter	12K
BBa_R0011	LacI promoter	pSB1A2	Regulatory	6G
G00100	Forward primer for sequencing/amplifying BioBrick parts	-	Primer	To be ordered
G00101	Backward primer for sequencing/amplifying BioBrick parts	-	Primer	To be ordered

[2] http://partsregistry.org/Part:BBa_J61002
[3] Consists of: RBS - CDS - double Terminator
[4] Each destination plasmid contains the mRFP1 as a control.

2. PROJECT OVERVIEW

2.1. GENES

The project will produce a set of BioBricks based on genes from various organisms (table 3). All BioBricks will be expressed in *E.coli.* In table 3 an overview is given of all the genes we will be incorporating into BioBricks with which *E.coli* K12 will be transformed. In the column headed "microorganism" the original microorganism from which the genes were isolated is given. We will obtain these genes through synthesis.

TABLE 3 – GENES TO BE USED

Gene	Feature	Microorganism
	Alkane degradation	
alkB2	Alkane 1-monooxygenase	*Gordonia* sp. TF6
rubA3	Rubredoxin 3 (transport chain enzyme)	*Gordonia* sp. TF6
rubA4	Rubredoxin 4 (transport chain enzyme)	*Gordonia* sp. TF6
rubB	Rubredoxin reductase (transport chain enzyme)	*Gordonia* sp. TF6
ladA	Alkane hydroxylase	*Geobacillus thermodenitrificans*
ADH	Alcohol dehydrogenase	*Geobacillus thermoleovorans*
ALDH	Aldehyde dehydrogenase	*Geobacillus thermoleovorans*
	Salt tolerance	
bbc1	Increases halotolerance of host	*Chlamydomonas* sp. W-80
	Emulsifier production	
AlnA	Emulsifying protein, homologue of the *E. coli* OmpA. (GenBank: AY033946.1)	*Acinetobacter radioresistens* (KA53)
OprG	Outer membrane emulsifying protein (NCBI Reference Sequence: NC_002947.3 from base 593454 to 594137)	*Pseudomonas putida* KT2440
	Hydrocarbon Sensing	
PalkS1-2	Prom: Contains AlkS binding site	*Pseudomonas putida* OCT
PalkB	Prom: Contains AlkS binding site	*Pseudomonas putida* OCT
P(CaiF)	Prom: Contains Crp binding site	*Escherichia coli*
AlkS	CDS: Transcription factor	*Pseudomonas putida* OCT
	Solvent Tolerance	
PhPFD-alpha	CDS: Prefoldin subunit alpha	*Pyrococcus horikoshii*
PhPFD-beta	CDS: Prefoldin subunit beta	*Pyrococcus horikoshii*

2.2. STRAINS

The most used strain in this project will be ***Escherichia coli* K12**; we will be using this as the host for the BioBricks. Furthermore we would like to use ***Pseudomonas putida* OCT1** as positive control in a number of characterization steps. This strain will not be modified in any way, and will just be used for comparison purposes. All the strains will be cultivated on solid medium plates or in liquid medium in shake-flasks with a volume of maximally 250 mL.

2.3. MATERIALS

HYDROCARBONS

For the characterization of the alkane degradation capacities as well as the emulsifier capabilities we will be using a number of different hydrocarbons:

- Octane, decane, undecane, dodecane, hexadecane, heptadecane and icosane, cyclohexane

CULTURE MEDIA

As culture media we will be using Luria-Bertani (LB) medium or M9 medium with the appropriate antibiotic. We will also be using a modified version of the M9 medium, in which there will be no glucose, but hydrocarbons instead.

EQUIPMENT

We will be using the following equipment:

- Gas Chromatograph (alkane measurements)
- Nanodrop (DNA+RNA measurements)
- Spectrophotometer
- Microwell-plate analyzer (Fluorescence)
- Shake-flasks, shake-bath etc. (cultivation)
- Gel-electrophoresis equipment (for SDS PAGE & Agarose)
- PCR

3. ALKANE DEGRADATION

3.1. AIM

Formation of BioBricks for the degradation of n-alkanes to n-alkanols followed by the conversion to n-alkanals and finally n-alkanoic acids. These biobricks will be implemented in *Escherichia coli* K12 and characterized and evaluated on their alkane degrading capabilities.

3.2. PROPOSED METHOD

The first sub-project will be the degradation of alkanes; here we will attempt to create an *Escherichia coli* K12 strain capable of converting medium (C_{5-13}) and long chain alkanes (C_{15-36}) into alkonoic-acids (figure 1). This will be accomplished by implementing specific genes into the strain using the "BioBrick" method. Once alkonoic-acids have been formed *E.coli* can further utilize them by converting them through β-oxidation.

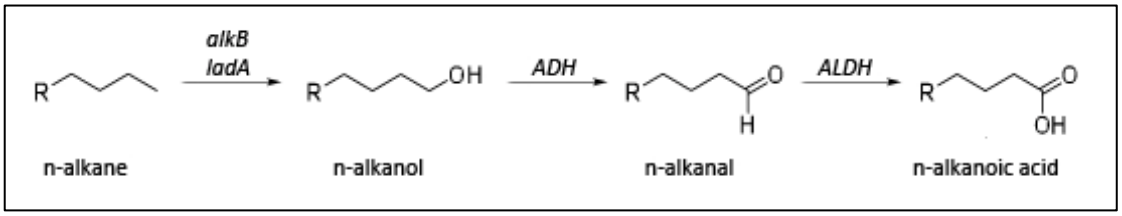

FIGURE 1 – STEPS AND ASSOCIATED ENZYMES FOR THE DEGRADATION OF ALKANES TO ALKANOIC ACIDS.

STEP 1: *AlkB,* FROM ALKANES TO ALKANOLS (C_{5-13})

***Based on**:* Fujii, T., Narikawa, T., Takeda, K., Kato, J., Biotransformation of various alkanes using the Escherichia coli expressing an alkane hydroxylase system from *Gordonia* sp. TF6. *Bioscience, biotechnology, and biochemistry*, **68**(10) 2171-2177 (**2004)**

- **Gene(s)**: alkB gene cluster (containing genes alkB2, rubA3, rubA4 and rubR) (to be synthesized)
  Originally from *Gordonia* sp. TF6
- **Vector:** pSB3C5 (medium copy, provided by iGEM organization)
- **Strain:** *Escherichia coli* K12
- **Final product:**
  Strain: *E. coli* K12/014C
  Resistance: Chloramphenicol
  BioBrick: BBa_K398014
  Contents: Promoter, RBS-1, alkB2, RBS-2, rubA3, RBS-3, rubA4, RBS-4, rubR
- **Cultivation:** 250 ml shake-flasks / solid agar plates

The alkB gene cluster from *Gordonia* sp. TF6 facilitates the initial step of the degradation of C_{5-13} alkanes as well as that of C_{5-8} cycloalkanes. The cluster is formed by the genes for *alkB2* (alkane 1-monooxygenase), *rubA3* (rubredoxin), *rubA4* (rubredoxin) and *rubB* (rubredoxin reductase). *alkB2* is a non-haem diiron monooxygenase membrane protein, reported for several genus and species. This monooxygenase oxidizes n-alkanes to the respective n-

alkanols and requires three soluble electron-transfer proteins, rubredoxin (*rubA3* & *rubA4*) and rubredoxin reductase (*rubB*).

The necessary genes, alkB2, rubA3, rubA4 and rubR, will be synthesized, and the appropriate RBS's and promoters that will be used are existing BioBricks. The transformed cells will be cultivated in shake-flasks of 250ml. The selection criterion for RBS and Promoter for each construction will depend on the results obtained during the RBS characterization experiments. The final construct will contain the alkB gene cluster, the necessary promoters and RBS's on a pSB3C5 plasmid. This construct, as well as all those in between, can be found in appendix A.

STEP 2: *LadA,* FROM ALKANES TO ALKANOLS (C_{15-36})

***Based on**:* Liu Li, Xueqian Liu, Wen Yang, Feng Xu, Wei Wang, Lu Feng, Mark Bartlam, Lei Wang and Zihe Rao. Crystal Structure of Long-Chain Alkane Monooxygenase (LadA) in Complex with Coenzyme FMN: Unveiling the Long-Chain Alkane Hydroxylase. *Journal of molecular biology*, **376**: 453–465 **(2008**)

- **Gene(s):** LadA gene (to be synthesized)
  Originally from *Geobacillus thermodenitrificans* NG80-2
- **Vector:** pSB3T5 (medium copy, provided by iGEM organization)
- **Strain:** *Escherichia coli* K12
- **Final product:** *Strain:* *E. coli* K12/016T
  Resistance: Tetracycline
  BioBrick: BBa_K398016
  Contents: Promoter, RBS-1, ladA
- **Cultivation:** 250 ml shake-flasks / solid agar plates

LadA, a long-chain alkane monooxygenase from *Geobacillus thermodenitrificans* NG80-2, facilitates the conversion of long-chain alkanes (up to at least C_{36}) to the corresponding primary alcohols. It will be used complementary to the *alkB2* cluster described in step 1.

The synthesized LadA sequence, and appropriate RBS and promoter from the BioBrick kit (existing biobricks) will be combined into one Biobrick. The transformed cells will be cultivated in shake-flasks of 250ml. The selection criterion for RBS and Promoter for each construction will depend on the results obtained during the RBS characterization experiments. The final construct will contain the LadA, the necessary promoters and RBS's on a pSB3T5 plasmid.

STEPS 3: ADH-ALDH, FROM ALKANOLS TO ALKANALS FOLLOWED BY ALKANOIC ACIDS

Based on: Tomohisa Kato, Asuka Miyanaga, Mitsuru Haruki, Tadayuki Imanaka, Masaaki Morikawa & Shigenori Kanaya. Gene Cloning of an Alcohol Dehydrogenase from Thermophilic Alkane-Degrading *Bacillus thermoleovorans* B23. *Journal of Bioscience and Bioengineering* **91**(1):100-102 **(2001)**

and

Tomohisa Kato, Asuka Miyanaga, Shigenori Kanaya, Masaaki Morikawa. Gene cloning and characterization of an aldehyde dehydrogenase from long-chain alkane-degrading *Geobacillus thermoleovorans* B23. *Extremophiles* **14:**33-39 **(2010)**

- **Gene(s):** ADH and ALDH (to be synthesized)

LadA insert from step 2
Originally from *Bacillus thermoleovorans* B23

- **Vector:** pSB3T5 (medium copy, provided by iGEM organization)
- **Strain:** *Escherichia coli* K12
- **Final product:** *Strain:* *E. coli* K12/021T
  Resistance: Tetracycline
  BioBrick: BBa_K398021
  Contents: Promoter, RBS-1, ladA, RBS-2, ADH, RBS-3, ALD
- **Cultivation:** 250 ml shake-flasks / solid agar plates

ADH, an alcohol dehydrogenase isolated from *Bacillus thermoleovorans* B23, is capable of converting n-alkanols into the corresponding n-alkanal, the second step in the biodegradation of alkanes. From this same microorganism we will also be using ALDH, an aldehyde deyhydrogenase that facilitates the third step in alkane degradation, from n-alkanals to n-alkanoic acids, which can then be further degraded through β-oxidation.

The synthesized ADH and ALDH sequences, as well as the LadA insertion from step 2, and appropriate RBS's and promoter from the BioBrick kit (existing biobricks) will be combined into one Biobrick. The transformed cells will be cultivated in shake-flasks of 250ml. The selection criterion for RBS and Promoter for each construction will depend on the results obtained during the RBS characterization experiments. The final construct will contain the LadA, ADH, ALDH and the necessary promoters and RBS's on a pSB3T5 plasmid.

STEP 4: COMBINATION & FINAL CONSTRUCT

- **Gene(s):** AlkB cluster (alkB2, rubA3, rubA4 and rubR) BioBrick from step 1
  LadA, ADH and ALDH BioBrick from step 3
- **Vector:** pSB3C5 & pSB3T5 (medium copy, provided by iGEM organization)
- **Strain:** *Escherichia coli* K12
- **Final product:** *Strain:* *E. coli* K12/021T-014C
  Resistance: Tetracycline & Chloramphenicol
  BioBricks: BBa_K398014 & BBa_K398021
  Contents: - Promoter, RBS-1, alkB2, RBS-2, rubA3, RBS-3, rubA4, RBS-4, rubR
  - Promoter, RBS-1, ladA, RBS-2, ADH, RBS-3, ALD
- **Cultivation:** 250 ml shake-flasks / solid agar plates

As the fourth step for the alkane degradation sub-part we will transform *E.coli* with both created plasmids, (1) the pSB3C5-AlkB BioBrick and (2) the pSB3T5-LadA-ADH-ALDH BioBrick. This should result in a strain that is capable of converting alkanes to alkanoic acids, which can then be further degraded by the β-oxidation pathway that is already present in *E.coli.*

STEP 5: CHARACTERIZATION

- **Strains:**
  - AlkB: *E.coli* K12/014C
  - LadA: *E.coli* K12/016T
  - LadA-ADH-ALDH: *E.coli* K12/021T

- AlkB-LadA-ADH-ALDH: *E. coli* K12/014C - 021T
- **Negative control**: *E.coli* K12
- **Positive control:** *Pseudomonas putida* OCT1

- **Cultivation:** 250 ml shake-flasks / solid agar plates

Characterization

The final step of the alkane degradation sub-project will be the characterization of the product strain (step 4), as well as that of the "in-between" strains created in steps 1, 2 and 3 (4 strains in total). The positive control will be an organism that can already degrade alkanes naturally, either *Pseudomonas putida*. The negative control will be an unmodified *E.coli* K12 without alkane degrading capabilities. The strains will be grown on low concentrations (1% v/v) of certain hydrocarbons, a choice still has to made between octane, decane, undecane, dodecane, hexadecane, heptadecane, icosane and cyclohexane. Growth curves will be followed using OD-600nm measurements and alkane concentrations as well as any products will be analyzed using gas chromatography. Next to growth-phase kinetics, enzyme activities within cell extracts will be measured.

4. SALT TOLERANCE

4.1. AIM

To create BioBricks that will facilitate an increased halotolerance against NaCl in the host organism (*E.coli*). These BioBricks will be implemented in *Escherichia coli* K12, characterized and evaluated on halotolerance as well as any influential effects on protein expression.

4.2. PROPOSED METHOD

This sub-project will focus on creating an *Escherichia coli* K12 strain which is capable of withstand the high salt (NaCl) concentrations present in the world's oceans. This will be done by implementing the bbc1 gene (from *Chlamydomonas* sp. W-80) in *E.coli,* using the "BioBrick" method. Once this has been achieved the effects of bbc1 on the halotolerance of the strain will be characterized as well as the effects of bbc1 on the production of other proteins (using GFP).

Bbc1 is an Algal protein, which has a high number of homologues in multiple species. Although the exact function is not know it has been shown to have an influence on the increase the halotolerance of *Chlamydomonas* sp. W-80. It has been successfully expressed in *E.coli,* and was shown to increase the salt-tolerance significantly (Tanaka *et al.* 2001).
***Based on**:* Satoshi Tanaka, Kazunori Ikeda, Hitoshi Miyasaka. Enhanced Tolerance Against Salt-Stress and Freezing-Stress of *Escherichia coli* Cells Expressing Algal *bbc1* Gene. *Current Microbiology*. **42**:173–177(**2001**)

STEP 1: BIOBRICK FORMATION

- **Gene(s)**: bbc1 (to be synthesized)
  Originally from *Chlamydomonas* sp. W-80
- **Vector:** pSB3T5 (medium copy, provided by iGEM organization)
- **Strain:** *Escherichia coli* K12
- **Final product:**

Strain 1:	*E. coli* K12/100T
Resistance:	Tetracycline
BioBricks:	BBa_K398100
Contents:	Promoter (strong), RBS-1, bbc1, TT
Strain 2:	*E. coli* K12/101T
Resistance:	Tetracycline
BioBricks:	BBa_K398101
Contents:	Promoter (moderate), RBS-1, bbc1, TT
Strain 3:	*E. coli* K12/102T
Resistance:	Tetracycline
BioBricks:	BBa_K398102
Contents:	Promoter (weak), RBS-1, bbc1, TT

- **Cultivation:** 250 ml shake-flasks / solid agar plates

The necessary gene, bbc1, will be synthesized, and the appropriate RBS and promoter that will be used are existing BioBricks. These components will be combined with a pSB3T5 plasmid. Three plasmids will be made, one containing the BBa_J23119 promoter, one containing the BBa_J23116 and one containing BBa_J23107. Where BBa_J23119 has a strong transcription level and BBa_J23107 has a (relatively) medium transcription level and BBa_J23116 has a low transcription level. This will allow us to detect the effects of differing concentrations of bbc1 on salt tolerance as well as the production of other proteins. The transformed cells will be cultivated in shake-flasks of 250ml. The final constructs (3) will contain the bbc1 gene under a high transcription level promoter (construct 1, BBa_J23119) as well as a low(er) transcription level promoter (construct 2, BBa_J23107 and construct 3, BBa_J23116), and the necessary RBS on (medium copy) pSB3T5 plasmids.

STEP 2: SALT TOLERANCE CHARACTERIZATION

- **Strains:**
  - bbc1 (high transcription): *E.coli* K12/100T
  - bbc1 (medium transcription): *E.coli* K12/101T
  - bbc1 (low transcription): *E.coli* K12/102T
  - **Negative control**: *E.coli* K12
- **Cultivation:** 250 ml shake-flasks / solid agar plates

In order to characterize the BioBricks, the strains containing one of the BioBricks will be cultivated under different salt concentrations. Of each strain, 4 cultures of 100ml will be characterized, each containing different salt concentrations, ranging from 0M up to 0.8M NaCl. OD_{600} measurements will be done in order to determine the growth curve and SDS PAGE will be used to show whether the protein is being produced.

5. EMULSIFIER PRODUCTION

5.1. AIM

To create BioBricks for the production of emulsifiers AlnA and OprG. These biobricks will be implemented in *Escherichia coli* K12 and characterized and evaluated on their emulsifying capabilities.

5.2. PROPOSED METHOD

In order to overcome the mass-transfer limitations of an oil-water environment, the production of an emulsifier is proposed. The presence of an emulsifier will increase the amount of alkanes dissolved in the water phase, increasing the availably for the microorganisms. Two emulsifiers from naturally oil-degrading bacteria will be expressed in *E.coli* K12 using the BioBrick method.

STEP 1: ALNA BIOBRICK FORMATION

***Based on**:* Gil Walzer, Eugene Rosenberg and Eliora Z. Ron. The Acinetobacter outer membrane protein A (OmpA) is a secreted emulsifier. *Environmental Microbiology* **8(6)**: 1026–1032 **(2006)**

- **Gene(s)**: *AlnA* (to be synthesized)
  Originally from *Acinetobacter radioresistens* (KA53)
  LacI promoter (BioBrick provided; BBa_R0034), RBS (BioBrick provided BBa_B0034) and Double forward terminator sequence (BioBricks provided BBa_0015)
- **Vector:** pSB1T3 (high copy, provided by iGEM organization)
- **Strain:** *Escherichia coli* K12
- **Final product:** *Strain 1:* *E. coli* K12/200C
  Resistance: Tetracycline
  BioBricks: BBa_K398200
  Contents: Promoter, RBS-1, AlnA, terminator
- **Cultivation:** 250 ml shake-flasks / solid agar plates

Oil-degrading strains of *Acinetobacter* produce a variety of high-molecular-weight biosurfactants. One of the best-studied emulsifiers is Alasan produced by *Acinetobacter radioresistens* (KA53). It contains three proteins of 15 kDa, 31 kDa and 45 kDa. The 45 kDa protein is associated with the major emulsifying ability and is called AlnA. AlnA is a homologue of the *E. coli* OmpA.

The necessary gene, AlnA, will be synthesized, and the appropriate RBS and promoter that will be used are existing BioBricks. The transformed cells will be cultivated in shake-flasks of 250ml. The selection criterion for RBS and promoter for each construction will depend on the results obtained during the RBS characterization experiments.

STEP 2: OPRG BIOBRICK FORMATION

***Based on**:* Gil Walzer, Eugene Rosenberg, Eliora Z. Ron. Identification of outer membrane proteins with emulsifying activity by prediction of β-barrel regions. *Journal of Microbiological Methods* **76**:52–57 **(2009)**

- **Gene(s)**: OprG (to be synthesized)
  Originally from *Pseudomonas putida* KT2440.
- **Vector:** pSB3C5 (medium copy, provided by iGEM organization)
- **Strain:** *Escherichia coli* K12
- **Final product:** *Strain 1:* *E. coli* K12/201C
  Resistance: Chloramphenicol
  BioBricks: BBa_K398201
  Contents: Promoter, RBS-1, OprG
- **Cultivation:** 250 ml shake-flasks / solid agar plates

OprG is another emulsifier, an outer membrane protein originating from *Pseudomonas putida* KT2440. The necessary gene, OprG, will be synthesized, and the appropriate RBS and promoter that will be used are existing BioBricks. The transformed cells will be cultivated in shake-flasks of 250ml.

STEP 3: CHARACTERIZATION – EMULSIFING ACTIVITY

- **Strains:**
  - AlnA: *E.coli* K12/200C
  - OprG: *E.coli* K12/201C
  - **Negative control**: *E.coli* K12
- **Cultivation:** 250 ml shake-flasks / solid agar plates

In order to determine whether the emulsifiers are produced, if they are excreted or not, and how well they emulsify, a characterization procedure will be performed. This will consist of the production of the emulsifier (to be shown using SDS PAGE), and an emulsification test with cell-free supernatant, purified cell extract and purified whole cells (washed). The emulsification test will occur by creating an alkane/water bi-phase mixture and determining the absorbance before and after addition of the emulsifier, and/or by shaking the sample vigorously and measuring the height of both phases before and after.

6. HYDROCARBON SENSING

6.1. AIM

Creation of a BioBrick incorporating a regulatory mechanism controlled by alkane levels. High alkane levels will then be used to activate expression of specific genes involved in the alkane degradation. The BioBricks will be implemented in *Escherichia coli* K12 and characterized and evaluated on it regulating capabilities controlled by alkane levels.

6.2. PROPOSED METHOD

***Based on**:*

Canosa, I., J. M. Sanchez-Romero, et al. A positive feedback mechanism controls expression of AlkS, the transcriptional regulator of the *Pseudomonas oleovorans* alkane degradation pathway. *Molecular Microbiology* **35(4)**: 791-799 (**2000**)

Moreno, R., A. Ruiz-Manzano, et al. The Pseudomonas putida Crc global regulator is an RNA binding protein that inhibits translation of the AlkS transcriptional regulator. *Molecular Microbiology* **64(3)**: 665-675 **(2007)**

van Beilen, J. B., S. Panke, et al. Analysis of Pseudomonas putida alkane-degradation gene clusters and flanking insertion sequences: evolution and regulation of the alk genes. *Microbiology-Sgm* **147**: 1621-1630 **(2001)**

Weissenborn, D. L., N. Wittekindt, et al. (1992). "Structure and Regulation of the Glpfk Operon Encoding Glycerol Diffusion Facilitator and Glycerol Kinase of Escherichia-Coli K-12." *Journal of Biological Chemistry* **267**(9): 6122-6131.

Functionality

To control when the organism produces enzymes needed for the degradation of alkanes we will characterize an alkane sensing mechanism.

The sensing mechanism proposed is that of the *Pseudomonas putida* GPo1 OCT plasmid alk genes cluster[5] (figure 2). In the presence of alkanes, the AlkS transcriptional regulator activates the expression of its own gene, and that of *alkT*, from a promoter named *PalkS2*. This allows achieving AlkS levels that are high enough to activate the expression of the *alkBFGHJKL* operon from the *PalkB* promoter. AlkS recognizes C_5–C_{10} n-alkanes as effectors, but does not

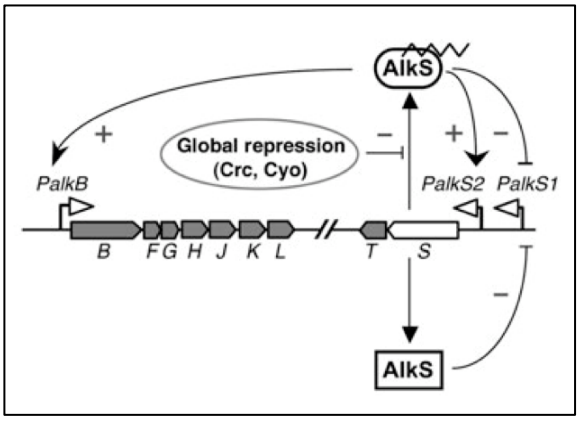

FIGURE 2 – ALKS HYDROCARBON REGULATORY SYSTEM FROM *PSEUDOMONAS PUTIDA*. (F. ROJO, *ET AL.* 2009)

[5] http://www.ncbi.nlm.nih.gov/nuccore/AJ245436

respond to shorter or larger alkanes (Rojo et al. 2009)

The Crp-protein is a global regulator ubiquitous to *E.coli* which is known to bind to regions of promoters known to activate genes involved in the degradation of non-glucose carbon sources, in this way activating the genes downstream of it. We would like to seamlessly integrate the expression of our alkane degrading genes into the *E.coli* system by making the alkane-degrading genes sensitive to Crp.

STEP 1: PalkS / AlkS BIOBRICK

Aim

Create a construct suitable for the analysis of transcriptional activity over the PalkS promoter unit and insertion thereof into competent E.coli K12.

- **Gene(s):** PalkS promoter unit (synthesized), AlkS translational unit (synthesized), GFP generator (BioBrick provided; BBa_E0240)
- **Vector:** pSB3C5 (provided by iGEM organization)
- **Strain:** *Escherichia coli* K12
- **Final product:** *Strain 1:* *E. coli* K12/310C
  Resistance: Chloramphenicol
  BioBricks: BBa_K398310
  Contents: PalkS, RBS-AlkS, GFP generator
- **Cultivation:** 250 ml shake-flasks (maximal volume) / solid agar plates

The first construct would be to analyze the activity of the PalkS1-2 promoter unit in the absence and presence of the active transcription factor, AlkS. AlkS is known to activate the alkane degradation genes operon (Alk genes) on the OCT plasmid in the presence of hydrocarbons. When AlkS binds to a hydrocarbon, it is activated and able to bind to the PalkS1 promoter region, inducing PalkS2 and increasing the transcription activity of its own gene, AlkS. In the absence of hydrocarbons, the AlkS transcription factor is inactive and its binding to PalkS1 represses transcription of the AlkS gene, thus keeping the AlkS protein at basal levels. In order to quantify these descriptions, a GFP translational unit will be placed behind the AlkS gene, thus making it dependent of the PalkS promoter unit. Fluorescence analyses in the absence and presence of hydrocarbons will provide us with the variations of AlkS transcription levels in the system.

STEP 2: PalkS / AlkS / PalkB BIOBRICK

Aim

To construct a plasmid containing the AlkS-PalkS-PalkB regulatory mechanism coupled to GFP and YFP generators in order to determine transcriptional activities of PalkS and PalkB at varying hydrocarbon concentrations by measuring fluorescence.

- **Gene(s):** PalkS promoter unit (synthesized), AlkS translational unit (synthesized), GFP generator (BioBrick provided; BBa_E0240), PalkB promoter unit (synthesized), YFP generator (BioBrick provided; BBa_E0422),
- **Vector:** pSB3C5 (provided by iGEM organization)
- **Strain:** *Escherichia coli* K12
- **Final product:** *Strain 1:* *E. coli* K12/311C
  Resistance: Chloramphenicol

BioBricks: BBa_K398311
Contents: PalkS, RBS-AlkS, GFP generator, PalkB, YFP generator

- **Cultivation:** 250 ml shake-flasks (maximal volume) / solid agar plates

After having determined the transcriptional activity profile of the PalkS promoter unit, an analysis will be made of the transcriptional activity of the PalkB promoter. For the sake of transformational simplicity the PalkB region will be placed downstream of the AlkS CDS, thus on the same plasmid containing Construct A. The transcription initiation activity of PalkB will be analyzed by placing a YFP generator downstream of it. By having both the GFP and YFP profiles proper analyses can be performed concerning AlkS levels and PalkB activity at varying hydrocarbon concentrations.

STEP 3: P(CAIF) / ALKS / PALKB BIOBRICK

Aim

The formation of a construct which facilitates the analysis of the transcriptional activity of the circuit responsive to hydrocarbons and to Crp.

- **Gene(s):** P(CaiF), GFP generator (BioBrick provided; BBa_E0240), AlkS (synthesized), PalkB (synthesized), glpR transcription factor (synthesized), Double forward terminator sequence (BioBrick provided; BBa_B0015), Synthetic RBS (BioBrick provided; BBa_0032)
- **Vector:** pSB3C5 (provided by iGEM organization)
- **Strain:** *Escherichia coli* K12
- **Product:** *E.coli* K12/312C
- **Cultivation:** 250 ml shake-flasks (maximal volume)

Having analyzed the transcription activities of the PalkB / AlkS promoter units the following step is the analysis of the Crp-protein regulation mechanism. Analysis of the GFP levels which are coupled to AlkS expression will give the information needed on functionality of the circuit.

STEP 4: CHARACTERIZATION

- **Strains:**
  - PalkS / AlkS: *E.coli* K12/310C
  - PalkS / AlkS / PalkB: *E.coli* K12/311C
  - P(CaiF) / AlkS / PalkB: *E.coli* K12/312C
  - **Negative control**: *E.coli* K12

- **Cultivation:** 250 ml shake-flasks / solid agar plates

The characterization process of the hydrocarbon sensing sub-project will entail output measurements of fluorescence at varying octane concentrations (0.1% – 1%) of the abovementioned strains. These measurements will be performed using a 96-well plate reader. Growth curves will be followed by OD-600 alongside the fluorescence measurements. The obtained fluorescence and growth measurements will be analyzed and modeled accordingly.

7. SOLVENT TOLERANCE

7.1. AIM

To transform *Escherichia coli* K12 and functionally express the protein prefoldin, which confers solvent resistance.

7.2. PROPOSED METHOD

***Based on**:* Okochi M., Kanie K., Kurimoto M., Yohda M. and Honda H.. Over expression of prefoldin from the hyperthermophilic arechaeum *Pyrococus horikoshii* OT3 endowed *Escherichia coli* with organic solvent tolerance. *Appl. Microbiol. Biotechnol*. **79**:443-449 **(2008)**

This sub-project will be the insertion and functional expression of a gene that confers solvent tolerance to *Escherichia coli* K12. All *Escherichia coli* strains are hydrocarbon sensitive, thus in order to make *Escherichia coli* cells capable of living on hydrocarbons they need to synthesize proteins that can make them resistant.
Prefoldin is a jellyfish-shaped hexameric chaperone that captures a protein-folding intermediate and transfers it to the group II chaperonin for correct folding. In this way it confers *E.coli* cells with resistance to hydrocarbons.

The sequences PhPFD-alpha, PhPFD-beta, appropriate RBS's and promoters will be required. The selection criterion for RBS and Promoter for each construction will depend on the results obtained during the RBS characterization protocol. Tentatively, the most used promoter will be BBa_J23109, which gives a medium to low transcription level and the most used RBS will be BBa_B0032 which gives a medium translation level.

STEP 1: FORMATION OF PREFOLDIN BIOBRICK

Aim: Creation of a BioBrick for the expression of both the alpha and beta subunits of prefoldin. A medium-transcription level promoter as well as a medium-translation level ribosomal binding site is utilized.

- **Gene(s)**: PhPFDα-RBS, pHPFDβ-TT (to be synthesized)
  Originally from *Pyrococus horikoshii* OT3
- **Vector:** pSB3C5 (medium copy, provided by iGEM organization)
- **Strain:** *Escherichia coli* K12
- **Final product:** *Strain:* *E. coli* K12/407C
  Resistance: Chloramphenicol
  BioBricks: BBa_K398407
  Contents: Promoter - RBS-1 - PhPFDα - RBS-2 - pHPFDβ-TT
- **Cultivation:** 250 ml shake-flasks / solid agar plates

STEP 2: CHARACTERIZATION

- **Strains:**

- Prefoldin: *E.coli* K12/407C
- **Negative control**: *E.coli* K12
- **Positive control:** *Pseudomonas putida* OCT

- **Cultivation:** 250 ml shake-flasks / solid agar plates

Characterization of ***E. coli* K12/407C** will involve its culturing on M9-modified liquid medium containing varying levels of cyclohexane (0%, 4%, 8%, 12% v/v). The positive control will be a colony of the *P. putida* OCT strain and the negative control will be an *E.coli* K12 colony, both grown under the same conditions. OD600 will be determined at various intervals between inoculation and 72 hours thereafter. Using these measurements the growth-related properties of each strain can be determined and analyzed accordingly.

8. CHARACTERIZATION OF THE ANDERSON RBS FAMILY

8.1. AIM

Formation of constructs facilitating the analysis of transcriptional activity over 5 different members of the Anderson RBS family by means of a GFP placed under the control of said RBS.

8.2. PROPOSED METHOD

In order to regulate the expression levels of the enzymes involved in the alkane degradation pathway we will characterize the Anderson family of ribosomal binding sites located in the distribution plates of Spring 2010. The final constructs (***BBa_K398022***; ***BBa_K398023***; ***BBa_K398024***; ***BBa_K398025***; ***BBa_K398026***) with which the *E.coli* K12 competent cells will be transformed will contain the relevant member of the Anderson RBS family, which have been supplied by iGEM, upstream of GFPmut3b. This translational unit will be placed downstream of a medium-strength promoter of the Anderson promoter family (http://2009.igem.org/Team:Groningen/Promoters).

Overall the formation of the final construct will involve three restriction reactions performed in parallel as well as one final ligation reaction of the acquired restriction products. The general methods will be in accordance with the general iGEM protocol for BioBrick Assembly (see **section 7.5**).

STEP 1: FORMATION OF RBS-GFP CONSTRUCTS

- **Gene(s)**: BBa_J61100 / BBa_J61101/ BBa_J61107 / BBa_J61117 / BBa_J61127
- **Vector:** pSB3C5 (medium copy, provided by iGEM organization)
- **Strain:** *Escherichia coli* K12
- **Final product:**

Strain 1:	*E. coli* K12/022C
Resistance:	Chloramphenicol
BioBricks:	BBa_K398022
Contents:	Promoter - J61100 – GFP –TT
Strain 2:	*E. coli* K12/023C
Resistance:	Chloramphenicol
BioBricks:	BBa_K398023
Contents:	Promoter - J61101 – GFP -TT
Strain 3:	*E. coli* K12/024C
Resistance:	Chloramphenicol
BioBricks:	BBa_K398024
Contents:	Promoter - J61107 – GFP –TT
Strain 4:	*E. coli* K12/025C
Resistance:	Chloramphenicol

BioBricks: BBa_K398025
Contents: Promoter - J61117 – GFP –TT

Strain 5: *E. coli* K12/026C
Resistance: Chloramphenicol
BioBricks: BBa_K398026
Contents: Promoter - J61127 – GFP -TT

- **Cultivation:** 250 ml shake-flasks / solid agar plates

STEP 2: CHARACTERIZATION

- **Strains:**
  - J61100 RBS: *E.coli* K12/022C
  - J61101 RBS: *E.coli* K12/023C
  - J61107 RBS: *E.coli* K12/024C
  - J61117 RBS: *E.coli* K12/025C
  - J61127 RBS: *E. coli* K12/026C
  - **Negative control**: *E.coli* K12

- **Cultivation:** 250 ml shake-flasks / solid agar plates

The characterization of the RBS sequences will involve determining their respective expression levels by means of fluorescence measurements of the strains during the growth phase. These measurements will be performed using a 96-well plate reader. Growth curves will be followed by OD600 alongside the fluorescence measurements. The obtained fluorescence and growth measurements will be analyzed and modeled accordingly.

PROJECT TIME LINE (TENTATIVE)

		RBS characterization	Alkane degradation	Salt tolerance	Emulsifier production	Solvent Tolerance	Hydrocarbon Sensing	Not Available	Other
week 1	June 7-11	BioBrick form. & characterization							
week 2	June 14-18	**Characterization & calibration** GC calibration, measure extraction efficiency, fluorometry, other analytical methods, Characterize *P.putida* and any other strains						H, T, P, N, K, L	Ethics Course
week 3	June 21-25							H, T, P, N, K, L	Ethics Course
week 4	June 28-2		BioBrick form.					K	
week 5	July 5-9		Characterization	BioBrick form.				K, H	
week 6	July 12-16			Characterization	BioBrick form.				
week 7	July 19-23				Characterization	BioBrick form.			
week 8	July 26-30					Characterization		T	
week 9	Aug. 2-6						BioBrick form.	T	
week 10	Aug. 9-13						Characterization		
week 11	Aug. 16-20								
week 12	Aug. 23-27								
week 13	Aug. 30-3								
week 14	Sep. 6-10								
week 15	Sep. 13-17								
week 16	Sep. 20-27								
week 17	Sep. 27-1								
week 18	Oct. 4-8								
week 19	Oct. 11-15								
week 20	Oct. 18-22								
week 21	Oct. 25-29								

H=Hugo, L=Luke, T=Thias, K=Kira, N=Nadine, E=Eva, P=Pieter, J=Jelmer, M=Mia, R=Ramon

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APPENDIX A: STRAIN TABLE (AND NOMENCLATURE)

Strain	Genes/biobricks	Resistance marker	Plasmid
	Alkane Degradation		
E. coli K12/007K	J23109, J61117	Kanamycin	pSB1K3
E. coli K12/008A	J23109, J61117, alkB2	Ampicillin	pSB1A3
E. coli K12/009K	rubA3, J61117	Kanamycin	pSB1K3
E. coli K12/010T	J61117, rubA3, J61117	Tetracycline	pSB1T3
E. coli K12/011K	rubA4, J61117	Kanamycin	pSB1K3
E. coli K12/012T	rubA4, J61117, rubR	Tetracycline	pSB1T3
E. coli K12/013K	J23109, J61117, alkB2, J61117, rubA3, J61117	Kanamycin	pSB1K3
E. coli K12/014C	J23109, J61117, alkB2, J61117, rubA3, J61117, rubA4, J61117, rubR	Chloramphenicol	pSB3C5
E. coli K12/015K	J23109, J61107	Kanamycin	pSB1K3
E. coli K12/016T	J23109, J61107, ladA	Tetracycline	pSB3T5
E. coli K12/017A	J23109, J61107, ladA	Ampicillin	pSB1A3
E. coli K12/018C	J61101, ADH	Chloramphenicol	pSB1C3
E. coli K12/019C	J61100, ALDH	Chloramphenicol	pSB1C3
E. coli K12/020A	J61101, ADH, J61100, ALDH	Ampicillin	pSB1A3
E. coli K12/021T	J23109, J61107, ladA, J61101, ADH, J61100, ALDH	Tetracycline	pSB3T5
	RBS characterization		
E. coli K12/022C	J23100-J61100-GFP-TT	Chloramphenicol	pSB3C5
E. coli K12/023C	J23100-J61101-GFP-TT	Chloramphenicol	pSB3C5
E. coli K12/024C	J23100-J61107-GFP-TT	Chloramphenicol	pSB3C5
E. coli K12/025C	J23100-J61117-GFP-TT	Chloramphenicol	pSB3C5
E. coli K12/026C	J23100-J61127-GFP-TT	Chloramphenicol	pSB3C5
	Salt Tolerance		
E. coli K12/100T	J23119-RBS-bbc1-TT	Tetracycline	pSB3T5
E. coli K12/101T	J23107-RBS-bbc1-TT	Tetracycline	pSB3T5
E. coli K12/102T	J23116-RBS-bbc1-TT	Tetracycline	pSB3T5
	Emulsifier production		
E. coli K12/200T	R0011-B0034	Tetracycline	pSB1T3
E. coli K12/201T	OprG-B0015	Tetracycline	pSB1T3
E. coli K12/202T	AlnA-B0015	Tetracycline	pSB1T3
E. coli K12/203K	R0011-B0034-OprG-B0015	Kanamycin	pSB1K3
E. coli K12/204K	R0011-B0034-AlnA-B0015	Kanamycin	pSB1K3
	Hydrocarbon Sensing		
E. coli K12/310C	*PalkS-RBS-AlkS-RBS-GFP-TT*	Chloramphenicol	pSB3C5
E. coli K12/311C	PalkS-RBS-AlkS-RBS-GFP-TT-	Chloramphenicol	pSB3C5

	PalkB-RBS-YFP-TT		
E. coli K12/312C	P(CaiF)-RBS-AlkS-TT-P(AlkB)-RBS-GFP-TT	Chloramphenicol	pSB3C5
E. coli K12/315K	PalkS-RBS-AlkS	Kanamycin	pSB1K3
E. coli K12/316T	PalkS-RBS-AlkS-RBS-GFP-TT	Tetracycline	pSB1T3
E. coli K12/317T	PalkB-RBS-YFP-TT	Tetracycline	pSB1T3
E. coli K12/318T	P(CaiF)-RBS	Tetracycline	pSB1T3
E. coli K12/319T	AlkS-TT	Tetracycline	pSB1T3
E. coli K12/320T	P(CaiF)-RBS-AlkS-TT	Kanamycin	pSB1K3
E. coli K12/321A	PalkB-RBS-GFP-TT	Kanamycin	pSB1A3
	Solvent tolerance		
E. coli K12/407C	J23109–B0032–PhPFDα–B0032-PhPFDβ-TT	Chloramphenicol	pSB3C5
	Strains with only existing Biobricks		
E. coli K12/X1A	J23109	Ampicillin	pSB1A2
E. coli K12/X2A	J61117	Ampicillin	pSB1A2
E. coli K12/X3A	J61107	Ampicillin	pSB1A2
E. coli K12/X4A	J61101	Ampicillin	pSB1A2
E. coli K12/X5A	J61100	Ampicillin	pSB1A2
E. coli K12/X6A	mRFP1	Ampicillin	pSB1A3
E. coli K12/X7K	mRFP1	Kanamycin	pSB1K3
E. coli K12/X8T	mRFP1	Tetracycline	pSB1T3
E. coli K12/X9C	mRFP1	Chloramphenicol	pSB1C3
E. coli K12/X10C	mRFP1	Chloramphenicol	pSB3C5
E. coli K12/X11T	mRFP1	Tetracycline	pSB3T5
E. coli K12/X12A	E0240	Ampicillin	pSB1A2
E. coli K12/X13A	E0422	Ampicillin	pSB1A2
E. coli K12/X14A	R0010	Ampicillin	pSB1A2
E. coli K12/X15A	B0032	Ampicillin	pSB1A2
E. coli K12/X16A	B0015	Amp/Kan	pSB1AK2
E. coli K12/X17A	I3401	Ampicillin	pSB1A2
E. coli K12/X18A	J61127	Ampicillin	pSB1A2
E. coli K12/X19A	R0011	Ampicillin	pSB1A2
E. coli K12/X20A	B0034	Ampicillin	pSB1A2