RISK ASSESSMENT

UANL_Mty-Mexico: Thermocoli

ORGANISM DESCRIPTION

Escherichia coli is a member of the family *Enterobacteriaceae* and has been described by Brenner (1984). *Escherichia* is a Gram-negative rod which can be motile by peritrichous flagella or nonmotile. *Escherichia* is also a facultative anaerobe which has both a respiratory and a fermentative type of metabolism, and commonly occurs in the intestinal tract of humans and other animals. The strain *E. coli* K-12 is a debilitated strain which does not normally colonize the human intestine. It has also been shown to survive poorly in the environment, has a history of safe commercial use, and is not known to have adverse effects on microorganisms or plants.

Because of its wide use as a model organism in research in microbial genetics and physiology, and its use in industrial applications, *E. coli* K-12 is one of the most extensively studied microorganisms. As a result, these K-12 strains are well-characterized and should be expected to remain as pure cultures under standard microbiological practices. K-12 strains are distinguishable from other Escherichia.

According to the Final Risk Assessment of the U.S. Environmental Protection Agency (EPA) (1997), a potential hazard of Escherichia coli K-12 and its derivative strains to human health should be addressed in terms of its potential to colonize the human intestinal tract and the production of a toxin. In this assessment, it was determined that E. coli K-12 does not pose a threat to human or animal health in terms of colonization; any concerns can be discarded because of this kind of bacteria's poor ability to survive and mobilize plasmids (to the native intestinal flora in murine models and even humans (Levy and Marshall, 1981). These same results have been observed in chickens and pigs (Smith et al., 2010).

○ Genetic modifications (design)

The project "*Thermocoli*" consists in a circuit of transcription factors and reporter genes, some of which are under the post-transcriptional regulation of thermoregulable RNA elements, also known as RNA thermometers. These genes are arrange in a circuit constructed in such a way that three distinguishable states should emerge, characterized by the expression and repression of two different reporter fluorescent proteins.

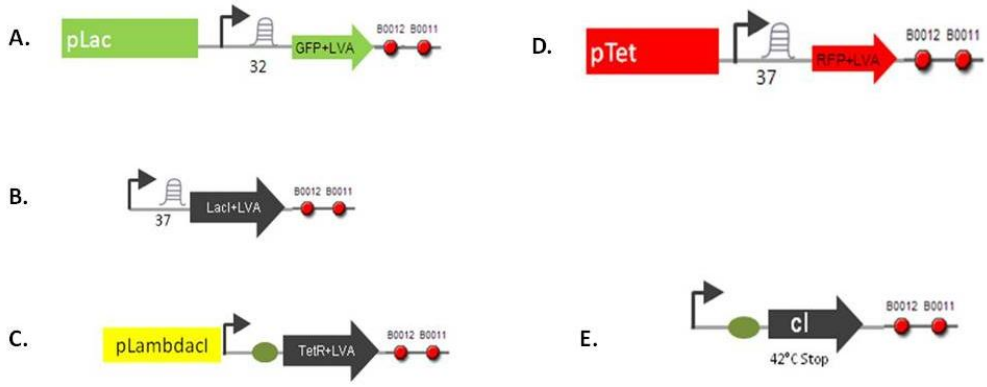


Figure 1. The submodules of the iGEM UANL 2013 project. Description in text.

In this work, we intend to regulate the expression not only reporter proteins, but also the expression of at least one transcription factor using RNA thermometers.

If proved possible, the RNA-thermoregulation of transcription factors will widen the spectrum of genetic circuit topologies that can be used for a number of purposes, most remarkably, the research of basic cellular processes and the replacement of chemical inducers for industrial-scale processes.

Our system can be subdivided in five different modules that can be characterized separately. Figure 1 shows those submodules labeled from A to E. Here we describe each one of them:

A. This synthesized construction has a gene that codes for the green fluorescent protein **(GFP)** variant that has an LVA degradation tag; the gene is under the regulation of a pLac promoter and a thermolabile ribosome binding site (RBS) that should allow for translation only at temperatures above 32°C. At the 3' end of the gene dubbed "GFP-LVA" are two consecutive transcription termination regions.

   The GFP sequence is similar to the one found in part **BBa_K145015** and is a mutant derived from the green fluorescent protein found in jellyfish (Andersen et al., 1998).

   The LVA tag that is in frame with the coding sequence of the TetR gene, is actually a derivation of the C-terminal AANDENYALAA tagging sequence, which makes proteins susceptible to degradation by the ClpX and ClpA proteases (McGiness, Baker and Sauer, 2006). After testing different variants for the last three amino acid residues in the C-terminal part of the peptide, it was found that AANDENYALAA variants show different degradation rates. We chose the LVA because is the most commonly employed in iGEM projects and we attached it to the coding sequence of GFP using a spacer (or *scar*) similar to the one present in part **BBa_J04450.**

   The transcription termination sequences employed are two: part **BBa_B0010** (derived from T1 of *E. coli* rrnB) and part **BBa_B0012** (derived from TE of bacteriophage T7). Both sequences are stem loops that hinder the processivity of E. coli RNA polymerase.

B. A construction that has a gene coding for the LacI transcription factor, which also has a LVA degradation tag. The LacI gene is transcribed through a constitutive promoter and a thermolabile RBS with an optimal translation temperature of 37°C. Two termination sites were added at the 3' end of the gene.

The constitutive promoter corresponds to the sequence of part **BBa_J23119**, the most potent member of a family of promoter variants obtained from a combinatorial library.

Transcription factor LacI is the same one found in *E. coli* lac operon and the sequence employed is the same as the one from part **BBa_C0012**, which is the same as the one employed in the Elowitz and Leibler 2000 study as stated in the "design" section of the registry entry for part **BBa_C0012**, this LacI variant differs from the wild-type in that the GTG start present in nature was changed to an ATG start.

C. This is a synthesized construction that also codes for an LVA-tagged transcription factor, which in this case is protein TetR. The gene is under the regulation of a pcI promoter, which is in turn regulated by transcription factor cI and a generic (non-thermolabile) RBS.

   The pcI promoter is derived from the pR promoter of bacteriophage lambda and is registered as **BBa_R0051** in the biological parts registry.

   The RBS sequence is derived from part **BBa_B0034**, which features in the 2010 study by Elowtiz and Leibler. This RBS is defined as the standard for RBS activity and is assigned an efficiency of 1.0.

   The coding sequence of TetR was derived from part **BBa_C0040**. TetR is a member of a family of transcriptional repressors present in gram-positive, alpha-,beta-, and gamma-proteobacteria, cyanobacteria and archea. The function of a TetR family member can be quite complex. However, the TetR sequence we are using should have a straightforward inhibiting activity upon the pTet promoter region, which is alleviated by the binding of tetracycline, or its analogue, aTc, to TetR. (Ramos, et al., 2005). The TetR sequence we are using is similar (as per a BLAST search) to other plasmids employed in Synthetic Biology projects.

D. A construction similar to A., but that has a red fluorescent protein-coding gene **(mCherry)** instead of GFP. This mCherry gene also has an in-frame degradation tag. However, the mCherry is under the regulation of a pTet promoter and a thermolabile ribosome binding site with an optimal translation temperature of 37 °C. Two transcription termination sites were also added at the 3' end of the gene.

The pTet promoter is the binding site for TetR, the transcription repressor, and it's identical to the sequence found in part **BBa_R0040**. Promoter pTet shows constitutive transcriptional activity until TetR binds to it. The sequences for TetR and the pTet promoter are similar to the ones present in *E. coli* Tn10 (tet) operon (Lutz and Bujard, 1997).

E. This construction is actually part BBa_K098995 and was the only construction which we didn't synthesize. It codes for a variant of *E. coli* transcription factor cI which is thermolabile. In principle, at 42°C this thermolabile cI should denature and stop its inhibiting action upon the pcI promoter. The cI gene is transcribed through a constitutive promoter and a generic (non-thermolabile) RBS. Two transcription termination sites were added at the 3' end of the gene.

- **Potential risks**

Given its natural habitat of the large bowel of mammals, *E*. *coli* will not likely survive for long periods in soil, water, or air. *E*. *coli* K-12 has been shown to have poorer survival characteristics in soil and water than other *E*. *coli*. The ability of *E*.*coli* to survive under environmental conditionsis thus very limited. *E*. *coli* K-12 has no known survival mechanisms in the environment, such as the ability to produce spores

E. coli is an inhabitant of the human colon, and it is thought that the primary means through which humans acquire their intestinal flora is through ingestion. Workers in fermentation facilities would not be expected to ingest *E. coli* under standard good practice, which prohibits the ingestion of food in work areas; however, some inhaled bacteria could be swallowed

As noted above, K-12 is defective in cell wall components relevant to the ability to recognize and adhere to the mucosal surface of colonic cells. The normal flora in residence in the colon thus can easily exclude K-12, and prevent it from colonizing the human colon in case of ingestion.

E. *coli* K-12 is defective in at least three cell wall characteristics:

a) The outer membrane has a defective lipopolysaccharide core which affects the attachment of the O-antigen polysaccharide side chains (Curtiss, 1978).

b) It does not have the type of glycocalyx required for attachment to the mucosal surface of the human colon as a result of the altered O-antigen properties noted above.

c) K-12 strains do not appear to express capsular (K) antigens, which are heat-labile polysaccharides important for colonization and virulence (Curtiss, 1978).

We discard any public and environmental safety concerns because we'll be working with typical laboratory strain (DH-5alfa), which have very low competitive advantages against wild-type microorganisms in the case of an accidental release. Appropriate

handling measures will be also applied for genetically modified bacteria and materials contaminated with bacteria.

As previously mentioned, K-12 strains are not pathogenic for humans, animals nor plants, and the genetic modifications made in the laboratory won't improve in this particular case the ability of the bacteria to affect the human health. Also none of the genes that will be employed (both, BioBricks and synthesized sequences) contain any sequence that shows resemblance to any known virulent agent.

LABORATORY

F. Workplace

For the use of chemical reagents and laboratory equipment we will follow the biosecurity rules imposed by Laboratory Biosafety Manual (3rd edition) – WHO, 2004:
www.who.int/csr/resources/publications/biosafety/CDS_CSR_LYO_2004_11SP.pdf

All the personal of the laboratory is aware of the biosafety rules, regulations and they comprehend the biosafety management plan. Also before using any kind of equipment for the first time, they have to read the manual and count with advisory.

These are some general guidelines that we follow in our laboratory:

a) The working area consist of level one laboratory on which only authorized personal and members of the team are allowed.

b) It uses to keep neat, clean and free for material that are not pertinent for the work.

c) The surface is decontaminated before start to work and before leaving.

d) All the material are cleaned after being used and the ones with biological material are decontaminated.

e) Doors and windows are closed at least it is necessary to open them.

f) There is always a copy of the operation manual in the laboratory.

g) The laboratory is well illuminated and is designed to be easy to keep clean and to be resistant.

h) There are some storage spaces are place inside the laboratory and other are outside, they are aisles adequate to hold the material and supplies.

i) Also a hand washing basins is placed in the laboratory.

j) A binnacle is used to control the activities done in the laboratory and follow the program. In case of emergency, the medical kit is always available.

k) There is an adequate electricity supply and emergency lighting to permit safe exit and to support essential equipment.

l) To enter in the laboratory is required to use laboratory lab, closed shoes, and the hair up if it is long.

m) Safety glasses, visors, gloves or other protective devices are worn when it is necessary. Also, all chemical and biological material is removed in special cans or decontaminated.

G. **Biocontainment**

Our project does not raise any safety issues other than the normally associated with working with typical cloning strains of the bacteria *Escherichia coli* and molecular biology procedures. Thus, in regard to researcher safety, our project doesn't pose risks that aren't contemplated in normal microbiology and molecular biology training for a BL-1 laboratory.

Work in the laboratory involves risks, but have taken action to minimize and prevent the accidental release and minimize risks:

a) Eating, drinking, smoking, handling contact lenses, applying cosmetics, and storing food for human consumption must not be permitted in laboratory areas. Food must be stored outside the laboratory area in cabinets or refrigerators designated and used for this purpose.

b) Decontaminate work surfaces after completion of work and after any spill or splash of potentially infectious material with appropriate disinfectant.

c) Persons must wash their hands after working with potentially hazardous materials and before leaving the laboratory.

d) Decontaminate all cultures, stocks, and other potentially infectious materials before disposal using an effective method. Depending on where the decontamination will be performed, the following methods should be used prior to transport.

e) The laboratory supervisor must ensure that laboratory personnel receive appropriate training regarding their duties, the necessary precautions to prevent exposures, and exposure evaluation procedures.

f) All students involved with the project were informed of the established security arrangement to strictly comply with the rules.

g) Also, prior to the beginning of the experimental procedures, all of the team members were given a course on biosafety, focused mainly on biological hazards, chemical reagents and general BSL-1 laboratory safety concerns and security procedures.

h) The proper use of containment equipment as well as personal protective equipment.

i) Materials to be decontaminated outside of the immediate laboratory must be placed in a durable, leak proof container and secured for transport.

Everybody is advised of special hazards with chemical substances of biological material, and the laboratory advisor make sure all members of the team follow the standard practices and procedures, and that they know what to do in case that an incident occurs

We are the first line of defense for protecting ourselves, others in the lab, and the public from exposure to hazardous agents. Protection depends on good microbiological practices and the correct use of safety equipment and through these procedures, none of the genetically modified bacteria should have a chance of being introduced into the environment. The constructs that we have built to test our systems in the laboratory all will use a safe, non-pathogenic bacterial strain of *E. coli* commonly used in labs worldwide.

- **Harzardous Substances Risk Assessment Summary Safety Sheets**

Sheet 1	Glacial Acetic Acid
Sheet 2	HCl
Sheet 3	Ethidium Bromide
Sheet 4	NaOH

STABILITY TEST

In the case of an accidental release (or even an intentional) of our plasmids, we'd be interested in knowing for how long will our cells keep the DNA with put on them, that is, how stable a plasmid is in the absence of a selective pressure, such as the antibiotic commonly used to specifically grow transformed cells. Plasmid stability tests are commonly employed to determine for how many generations can a cell culture keep a foreign plasmid in non-selective conditions.

We started with the assumption that our constructions -RNATs and reporter proteins- do not confer a selective advantage to the cells. In other words, we assumed that the replication origin of each plasmid should be the main factor that affects plasmid stability. The core plasmids we needed to test were pUC and pSB, since all of our constructions are based on this skeletons. However, due to time constrains, we were only able to test pUC.

We followed the protocol found in Bryksin and Matsumura, (2010), with a 100 mL culture that was re-inoculated with 100 µL of the culture of the previous day.

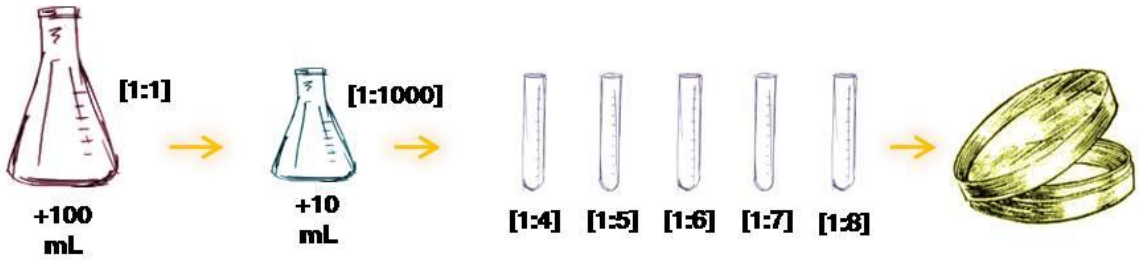
Plasmid stability test in E. coli:

- Inoculate a flask with 10 ml of LB medium with the proper antibiotic.

- Grow overnight until saturation is reached.
- Inoculate 100 mL of fresh LB without antibiotic with 100 µL of the overnight culture. Simultaneosuly, inocculate a dilution series (from 10^{-4} to 10^{-8}) of the overnight culture on two series of plaques, one with the antibiotic and one without.

- Repeat the procedure for eight days (approximately 80 generations, according to Bryksin and Matsumura, 2010). Nevertheless, we stopped the experiment when the culture reached 0% retention.

- Calculate each day the percentage of Colony Forming Units (CFUs) that retain the plasmid by dividing the number of cells growing in plaques with antibiotic by the number of cells growing without antibiotic.



We observed that after day 3 (generation 30), pUC showed a pronounced decrease on plasmid retention (from 30% to 1%) and reached 0% on day 5.

This allows us to conclude that, in the case of a release to the environment, when the retention of the plasmid is left to the effect of genetic drift, at least 30 generations will pass until almost no cell retains the plasmid pUC and its derivatives that harbor genes that do not confer a selective advantage.

Future work will be done to determine the stability of plasmid pSB and to let both plasmids grow after the day they reach 0% retention to see if the plasmid somehow re-emerges

IDENTIFICATION (ID Tags)

"Randy"

Due to the recent increase in synthetic biology projects with great potential for direct application in the environment, industry, or any other activity that requires the release beyond the laboratory, it is necessary to take apart biosecurity measures to allow growth only in conditions specific and desired, we ensure the tracking of such agencies in the event that at some point these genetic technologies fail.

UANL team aware of this need, has designed a proposal for screening and identification of genetically modified organisms. This proposal is the design of small sequences flanking the prefix and suffix of the synthetic constructs, unique to each team participating in iGEM, and that can be detected by a single PCR. Within the proposal also suggested that the primers required for the detection of genetically modified organisms of each institution participating in the iGEM be sent to the registry part so that they can be stored, and if necessary they can be easily distributed.

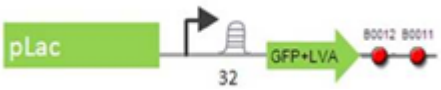
It is worth mentioning the importance and functions of these new "OMG ID Tag's" which are the:

1) **Comparison** between Synthetic Organism and Wildlife Organism.
2) **Identification** of synthetic organisms in nature are undesirable conditions which may affect the environment in which they are located.
3) **Possibility to contact** the organization where that synthetic organism was created to gather information needed to expedite a contingency plan.

- **PCR for detection**

This option consists in a simple detection of primer's design that will give us a specific size of band thanks to the arrangement of pieces. Although is the simplest way, it has issues being the main use of several primers for the detection of all the designed pieces. We are going to show the designed primers for this year pieces and information of interest like the melting temperature, the amplified piece and the size that will be created.

For the piece which is composed by **pLac + 32°C RNA thermometer + GFP (LVA)** [BBa_K1140002] we used the following primers:



Forward	gtccacacaatctgcccttt	Tm – 60º
Reverse	acccctcaagacccgtttag	Tm – 60º
		Product size: **203bp**

Fort he piece which is composed by **pCons + 37°C RNA thermometer + LacI (LVA)** [BBa_K1140002] we used the following primers:

Forward	gattggcgttgccacctc	Tm – 62º
Reverse	agcccggactcggtaatg	Tm – 61º
		Product size: **597bp**

Fort the piece composed by **pTet + 37°C RNA thermometer + RFP (LVA)** [BBa_K1140002] we used the following primers:

Forward	ttcatgcgtttcaaagttcg	Tm – 60º
Reverse	ggtggagtgacgaccttcag	Tm – 60º
		Product size: **639bp**

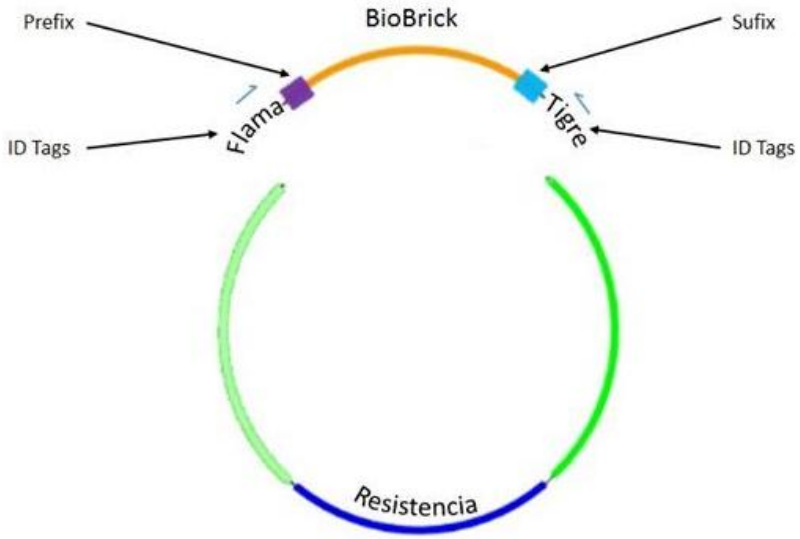
- **ID Tags – UANL**

The design of our "ID Tag's" consist of a pair of sequences, one upstream of the prefix and suffix other downstream, using a set of nucleotides with a meaning for us.

Prefix – ID Tag:	**tttctggcgatggcgGAATTCGCGGCCGCTTCTAGA**
If we translate the sequence **"tttctggcgatggcg" prefix-ID tag** will result us in spanish the word **"FLAMA"** giving reference to the motto of our university ALERE FLAMMAM VERITATIS	

Suffix - ID Tag:	**ACTAGTAGCGGCCGCTGCAGacaataggacgcgaa**
If we translate the sequence **"acaataggacgcgaa" suffix-ID tag** us will result in the spanish word **"TIGER"** referring to the mascot of our University.	

Basically, the idea consists in creating the plasmid pSB1C3 personalized for each university that will be participating, they will have their ID tags such that with those designed primers just an specific site will be amplified, in that way each team will have two sequences that will recognize their constructions.



Here we present the primers we used for this year **iGEM** contest:

		nt	GC	Tm
Forward	tttctggcgatggcgGAATTC	21	52.4%	58.3º
Reverse	ttcgcgacctattgtCTGCAG	21	52.4%	57.4º

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References:

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