iGEM FCB-UANL 2021

RISK ASSESSMENT

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LEGAL PERSPECTIVE

Mexico is part of two major international agreements: the Cartagena Protocol, which deals with biosafety issues, and the Nagoya-Kuala-Lumpur Protocol on Liability and Compensation. Both emerge from the Convention on Biological Diversity (CBD), which has other approaches for the conservation and sustainable use of biodiversity and the responsible use of genetic resources.

The function of these different protocols is to guarantee an adequate level of protection concerning the transfer, management, and use of living modified organisms (LMOs) of the current biotechnology application, which may affect the responsible use of biological diversity and its conservation.

These agreements promote different procedures to carry out safe handling, transfer, and use of LMOs. These aforementioned criteria are based on the analysis of possible future effects of LMOs and the extent of the damage if released before transfer. The Nagoya-Kuala Lumpur protocol is an international protocol that complements the Cartagena protocol contributing to the conservation and sustainable use of biodiversity. This protocol proposes to examine other previous criteria depending on the situation.

Evaluating other factors, such as risk to human health, is critical based on responsible international standards and procedures. This protocol aims to prevent, reduce, contain, and avoid the possible damage caused by living modified organisms (LMOs).

In addition, Mexico has different legal documents, such as The Law of Biosafety for Genetically Modified Organisms (LBOGM). Our government specifies the measures that must be taken into account when working with GMOs. Besides, this Law also establishes responsibilities for three regulatory organisms. In the case of our project, the primary regulatory organism is SEMARNAT.

PROTECTION OBJECTIVES

Human:

- Protecting the team's safety in the work environment with an influential culture of prevention.
- Ensuring that every member of the team involved with laboratory work is qualified to handle the material safely and are also prepared to handle possible risk scenarios related to the development of the project.
- Having a clear path of responsibilities within the laboratory, as well as a constant communication of the procedures and results obtained during lab work.
- Protecting the community's well-being by ensuring the isolation of the GMO and its genetic material, and also by avoiding the use of any pathogenic genes or species that may be harmful to humans, animals or plants.

Environment:

- Ensuring that the GMO and its genetic material remains contained within the limits of the laboratory setting.
- Preventing the gene-leaking caused by horizontal gene transfer.
- Avoiding the use of any pathogenic genes or the handling of species that may be harmful to humans, animals or plants.

Laboratory:

- Ensuring the appropriate use of the laboratory equipment by following standardized protocols.
- When possible, avoiding the use of harmful chemicals such as Ethidium bromide with safer alternatives such as GelRed; and when the use of a safer alternative is unfeasible, making sure that all of the preventive measures are being taken to prevent any harmful effects caused by the material.

ASSESSMENT OF THE POSSIBLE RISK ASSOCIATED WITH THE PROJECT

Brief description of the project:

The FCB-UANL iGEM team is developing a bio-inspired firefighting foam that seeks to replace the damaging, polluting fluorosurfactants present in most commercially available firefighting foams. To achieve our goal, the team plans to recombinantly produce in *Escherichia coli* a series of proteins called ranaspumins, which have surfactant and stabilizing properties. Moreover, additional surfactant and stabilizing agents will be

added in the form of surfactin and biofilm, respectively. They are both naturally produced by ***Bacillus subtilis***, to which we will apply principles of metabolic engineering to enhance the production of the previously mentioned biomolecules.

Description of the GMO

The project intends to generate four Genetically Modified Organisms (GMO). The details concerning each strain are described below.

- ***Escherichia coli* with Ranaspumin-2 expression gene:** Ranaspumin-2, a surfactant protein with no significant safety concerns reported, will be recombinantly expressed in ***Escherichia coli*** TOP10 via a vector system.
- ***Escherichia coli* with Ranaspumins-3-5 expression genes:** Ranaspumins 3 to 5, are stabilizing proteins with no significant safety issues reported, they will be recombinantly expressed in ***Escherichia coli*** TOP10 via a vector system.
- ***Bacillus subtilis* strain with enhanced surfactin production:** Key gene expression regulators involved in surfactin production, with no significant safety issues previously reported, will be synthesized through integration of a genetic circuit into the organism's chromosome.
- ***Bacillus subtilis* strain with enhanced surfactin production:** Key gene expression regulators involved in biofilm production, with no significant safety issues previously reported, will be synthesized through integration of a genetic circuit into the organism's chromosome.

PURPOSE OF THE GMO

General objective of the project:

- Designing an expression cassette that enhances the expression of surfactin and biofilm in ***Bacillus subtilis.***
- Designing an expression cassette to express Ranaspumin 2, 3, 4 and 5 in ***Escherichia coli.***

Short term objectives:

- Using synthetic biology techniques to avoid the sporulation of ***Bacillus subtilis***.
- Using synthetic biology techniques to synthetize the ranaspumin family of proteins (2 to 5) in ***Escherichia coli*** chassis.

- Using synthetic biology techniques to enhance the production of surfactin in *Bacillus subtilis*.
- Using synthetic biology techniques to maximize the production of biofilm in *Bacillus subtilis*.

Long term objectives:

- Developing a competitive eco-friendly firefighting foam composed of natural surfactant proteins.

IDENTIFICATION OF POTENTIAL HAZARDS OF THE GMO

The recipient organisms

***Escherichia coli* K12 Top 10**

Kingdom: Bacteria
Subkingdom: Negibacteria
Phylum: Proteobacteria
Class: Gammaproteobacteria
Order: Enterobacteriales
Family: Enterobacteriaceae
Genus: Escherichia
Species: *Escherichia coli*

Escherichia coli K12 Top10 is commercially available by Invitrogen. The strains derived from *E.coli* K12 are some of the most studied organisms in molecular biology, and they have been completely sequenced. Nowadays, because of its predictability and history of safe use, *E.coli* K12 variants are routinely used for studies in molecular biology as model organisms, and they are considered safe to work with. Derivatives of K12 are considered a-virulent (Hacker & Ott, 1992). They may be classified in the CDC biological agents hazard group 1 because these strains have no adverse effects on human, animal or plant health or the environment. Besides, there is an established safety record regarding their use in the laboratory (Tian & Tao, 2014). Furthermore, the *E.coli* strains used (Top10) are incapable of surviving outside the laboratory environment.

***Bacillus subtilis* ATCC 6633**

Kingdom: Bacteria
Subkingdom: Posibacteria

Phylum: Firmicutes
Class: Bacilli
Order: Bacillales
Family: Bacillaceae
Genus: Bacillus
Species: ***Bacillus subtilis***

Bacillus species are aerobic, rod-shaped bacteria that stain Gram-positive or Gram-negative (Cote ***et al.***, 2015). They form spores resistant to cold, heat, and common disinfectants, thus enabling the bacteria to survive in various environments (Cote ***et al.***, 2015). ***Bacillus subtilis***, the most widely studied Gram-positive bacterium, has long been used for biotechnology applications. It is considered a benign organism as it does not possess traits that cause disease. Therefore, it is not considered pathogenic nor toxigenic to humans, animals or plants. Besides, the potential risk associated with using this bacterium in fermentation facilities is low (EPA & of Pollution Prevention, 1997). ***B. subtilis*** is also widely used for the secretory expression of many industrial enzymes and pharmaceutical proteins. The ATCC 6633 strain is a commercial strain.

Both microorganisms are commercially available, and their biosafety level is 1. In other words, the experiments carried out with these agents are generally performed on standard open laboratory benches without the use of special containment equipment.

The donor organisms:

Note that NONE of the donor organisms was directly handled in the lab; only their sequence was used. The sequences of interest were synthesized by IDT.

Engystomops pustulosus

Kingdom: Animalia
Phylum: Chordata
Class: Amphibia
Order: Anura
Family: Leptodactylidae
Genus: Engystomops
Species: ***Engystomops pustulosus***

Engystomops pustulosus is found from Mexico and throughout Central America all the way into northern South America and as far east as Trinidad and Tobago, Venezuela, and

possibly Guyana. Its natural habitats are subtropical or tropical dry forest, dry savanna, moist savanna, subtropical or tropical dry lowland grassland, subtropical or tropical seasonally wet or flooded lowland grassland, freshwater marshes, intermittent freshwater marshes, pastureland, heavily degraded former forest, ponds, and canals and ditches. An interesting trait of ***Engystomops pustulosus*** is that it lays its eggs in foam nests, generally under cover in pools. The foam nests are believed to prevent egg desiccation during brief periods without rain (Ryan, 1985), and laying eggs under cover may help avoid predation (Tarano, 1998). In addition, eggs can be laid in a variety of habitats, including disturbed areas. However, pond use is correlated with proximity to other ponds, making this species vulnerable to habitat fragmentation (Marsh ***et al.,*** 1999).

THE INSERTS

Ranaspumin-2 (Rsn-2) gene is inserted in a pSB3K3 plasmid to be expressed in ***Bacillus subtilis***.

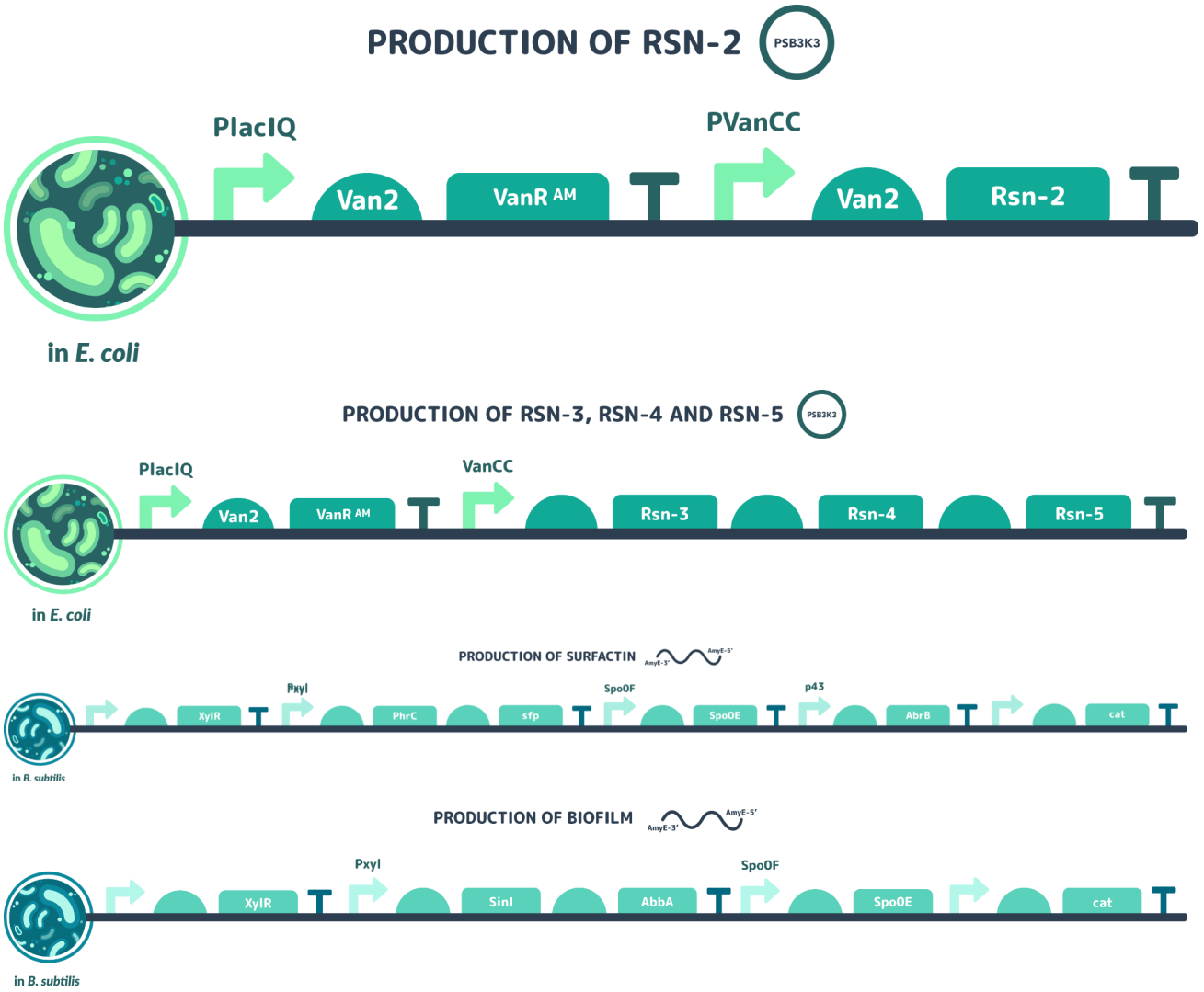

Figure 1. Genetic circuits for SYNBIOFOAM 2021 by team FBC-UANL 2021.

Ranaspumin-2 (Rsn-2) is a monomeric, 11 kDa surfactant protein identified as one of the major foam nest components of the túngara frog (***Engystomops pustulosus***), with an amino acid sequence unlike any other protein described so far.

VECTORS USED

The vectors used for this project are plasmids supplied by the iGEM organization. Both are pUC derived, and they do not possess severe pathogenic traits. However, they

present antibiotic resistances, pSB3K3 to Kanamycin and pS1C3 to Chloramphenicol, these resistances are for selection purposes and do not represent a risk.

TOXICITY OF THE RECOMBINANT SUBSTANCES

- None of the produced proteins (Ranaspumin-2, Ranaspumin-3, Ranaspumin-4 and Ranaspumin-5) to be used for this project are known to be involved in pathogenicity of their hosts.
- There is a slight concern involved with the overexpression of surfactin, Since its production promotes the formation of biofilm and has been mentioned as a potential virulence factor.
- None of the inserted genes in ***B. subtilis*** SinI, AbbA, Spo0E, cat, sfp, AbrB are virulence factors, however they do play physiological roles in their native hosts.

THE RESULTING GMO

During the first experimental phase of the project, the final products were four distinct GMOs in order to analyze the production of each of the fundamental components of the proposed fire fighting foam.

- The first is a non-pathogenic ***E. coli*** chassis containing an expression cassette designed to produce Ranaspumin-2, regulated with a vanillic acid-sensitive promoter. This component will be produced in an isolated manner since it is the active component of foam formation in the mixture.
- The second organism is a non-pathogenic ***E. coli*** chassis containing an expression cassette designed to produce Ranaspumin-3, Ranaspumin-4, and Ranaspumin-5, which act as stabilizing agents in the mixture.
- The third organism is a non-pathogenic ***B. subtilis*** chassis containing an expression cassette designed to overexpress surfactin. In this cassette, PhrC and Sfp genes will be regulated by xylose to increase surfactin production by promoting the production of proteins. In this same organism, the regulator AbrB will be used to decrease bacterial sporulation and biofilm production.
- The fourth organism is a non-pathogenic ***B. subtilis*** chassis containing an expression cassette designed to increase biofilm production by upregulating SinI and AbbA.

HAZARDS ASSOCIATED WITH PROJECT DEVELOPMENT

The possible hazards associated with the protection objectives of the modified bacteria under different parameters could be:

Environment

- Leakage of the modified bacterium into the environment through spores or other means.
- Horizontal and/or vertical gene transfer to other microbial populations.
- Possible pathogenic effects in other organisms under specific conditions.
- Invasive competitiveness of the modified bacteria upon the naturally found.
- Variants in the environment.

Human

- Pathogenicity in immunocompromised individuals.
- Possible human contact through aerosols.

Laboratory

- Leakage of the modified bacterium through physical medium (footwear, air, insects).
- Leakage of the bacterium through waste disposal areas of the laboratory.
- Leakage of the bacterium through accidental production of spores.

Risk of the activities that will be involved in this project:

The only living microorganisms handled by the team are non-pathogenic strains of *Escherichia coli* K-12 Top10 and *Bacillus subtilis* ATCC 6633 with low survivability outside laboratory conditions; therefore, the biological risk is very low. The experiments are standard molecular biology techniques, and the team is in compliance with iGEM's safety and security rules, policies and is supervised at all times by at least one of the two instructors of the team. There is also a subdivision within the team explicitly dedicated to monitor and assess any risks involved in the project.

Possible risks related to the chemicals associated with the GMO:

All the chemicals used for the production of the GMOs specified in this document have been approved to be used by our PI. Risks associated with the use of specific chemicals have been managed by the development of safety measures to handle all chemical substances.

Concentration and scale of the project:

For the experimental phase of the project, the GMO was cultured on LB solid media (20 Petri dishes at a time). The bacterial stock was stored and handled in 50 µL tubes. If the experiments prove to be a success, the project will be upscaled.

Culture conditions:

In the experimental phase, the GMOs were incubated at 37°C for 24 hours in LB broth with the addition of vanillic acid. These activities took place in a certified Level 1 BSL laboratory located in the facilities of the UANL. No wind currents exist within the lab that may cause aerosols to spread in the room, and all of the equipment has its designated area. The techniques that involved the handling of the organisms, like inoculation in medium and agar plates, took place in close proximity to a bunsen burner. All of the disposable used tools and plates were sterilized before appropriate disposal.

ENVIRONMENTAL CONCERNS OF THE GMO

Environment which is more likely to be in contact with the GMO

During the experimental phase, the only environment identified as a risk of being exposed to the GMO is the laboratory. However, it is not expected for the organism to survive outside containment (Miyanaga ***et al.,*** 2006).

Presence of susceptible species:

- Neither one of the strains, ***Escherichia coli*** K12 nor ***Bacillus subtilis*** ATCC 6633, that will be used as recipient represent a risk towards humans, animals or plants; and the expressed gene products of the inserts are not considered harmful or capable of altering the pathogenicity/survivability/fitness of the recipient organism.
- The strains of ***Escherichia coli*** K12 that will be used in the experiments have an extremely low survival rate outside containment (Miyanaga ***et al.,*** 2006). They also

lack pathogenicity, therefore lacking the ability to affect the immediate physical environment.

Survivability of the GMO

- The *Escherichia coli* strain used as chassis for the experimental phase by itself has a very low survival rate outside containment.
- The engineered *Bacillus subtilis* will contain a killswitch to prevent survival into the environment.

Effects of the GMO on the physical environment:

None of the by-products from the GMOs are harmful to the environment.

CONTAINMENT MEASURES

During wet lab procedures, the organism was contained inside a freezer within a BSL1 in the laboratory facilities of the "Facultad de Ciencias Biológicas" at the "Universidad Autónoma de Nuevo León". At all times, the organism was handled in sterile conditions, taking into account the appropriate microbiology procedures, following the methodologies laid out by our institution on this matter.

For the sole purpose of biocontainment, we designed a killswitch. For the construction of this circuit, we considered potential applications of our product/project in the future as a measure to fight fires in the environment.

PATH TO DAMAGE

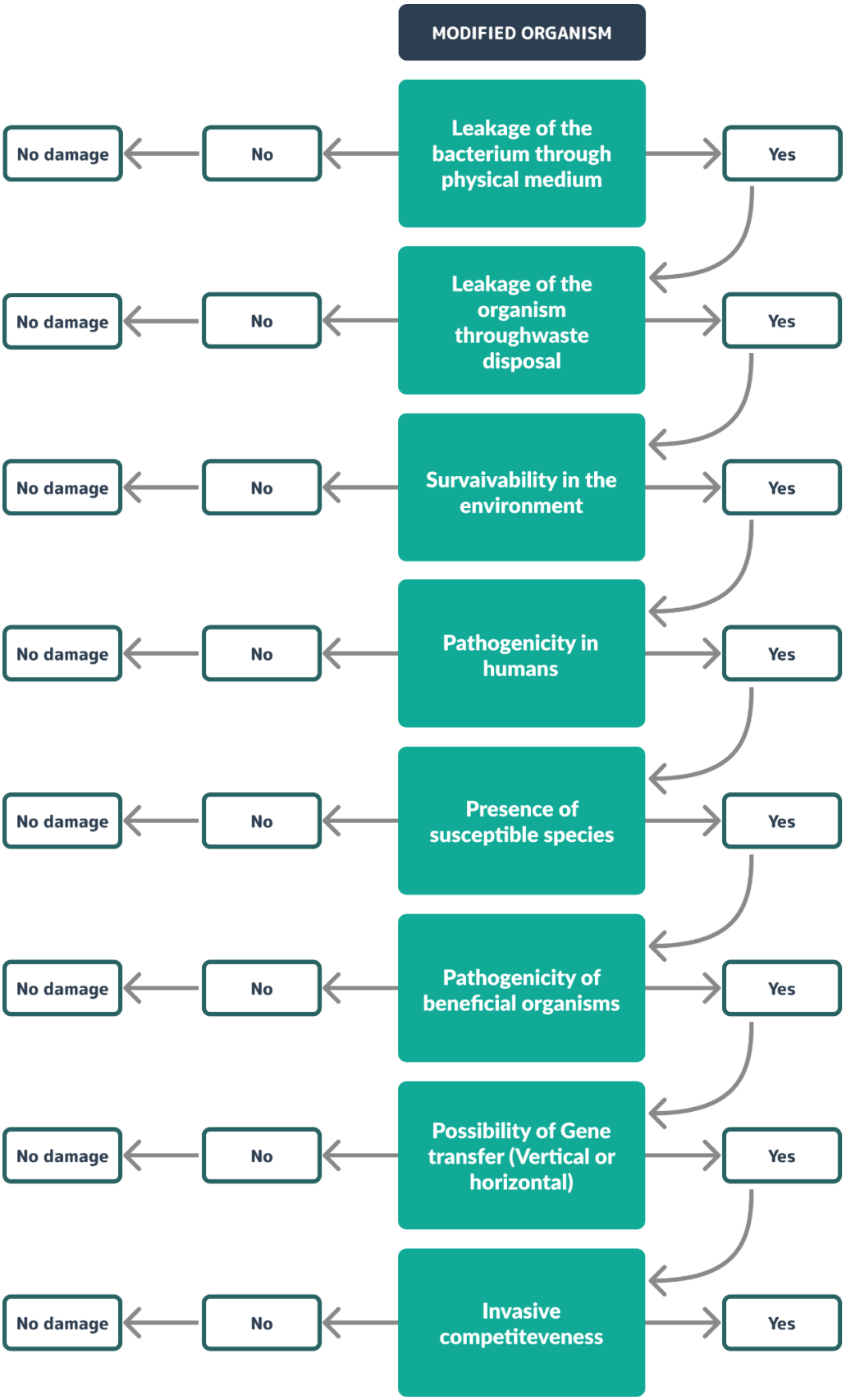

Figure 2. Path identified during this year in order for the risks to be present. This diagram aims to show the consequent steps that must happen in order for risk to be present.

RISK EVALUATION

Both *Escherichia coli* K12 Top10 and *Bacillus subtilis* ATCC 6633 have a history of being safe organisms in the laboratory. Therefore, it is unlikely to cause illness to humans, animals, or plants. Also, genetic modifications are not expected to alter these characteristics. The pUC derived vectors also have a history of safe use and are provided by the iGEM Organization. In addition to this, the inserts do not give the recipient organism characteristics that may cause disease or ill effects on the environment. Hence, the GMOs produced can be classified as Risk Level 1 organisms, which means that harmful effects are very low. Based on this, we estimate that the risk involved in this project is low. And taking into consideration the constant expert supervision and the strict safety measures taking place, the probability of an incident is very low, thus concluding that the possibility of risk is insignificant.

		RISK ESTIMATION			
PROBABILITY	VERY HIGH	LOW	MODERATE	HIGH	HIGH
	HIGH	LOW	LOW	MODERATE	HIGH
	LOW	INSIGNIFICANT	LOW	MODERATE	MODERATE
	VERY LOW	INSIGNIFICANT	INSIGNIFICANT	LOW	MODERATE
		MARGINAL	MINOR	INTERMEDIA	MAYOR
		CONSEQUENCE			

Figure 3. Risk evaluation based on the comparison of the probability for the risks identified during the risk assessment, versus the consequences that those risks could have on the surrounding environment.

RISK MITIGATION

Safe laboratory practices

- Dra. Lydia Guadalupe Rivera Morales is the executive secretary of our faculty's Biosafety committee, which is the organism in charge of overlooking all of the research that takes place in this institution and managing biosafety-concerning issues at the institutional level; however, the team has taken its measures in a smaller scale by creating a team sub-division focused in the safety of our project.

- Everyone involved in any work in the laboratory must have gone through a training of the do's and don'ts in the lab before getting involved in the work.
- At all times in the laboratory, the students involved in the team will be supervised by at least one of the instructors. Only the people who have had previous training in the use of lab equipment and safety protocols will be allowed to perform experiments.

Waste treatment procedures

- Accidental spillages were dealt with disinfectant 70% Ethanol.
- Contaminated, solid waste, such as plastic disposables, were bagged and autoclaved at 121°C for 20 mins, 1 bar pressure. The autoclave is located in the laboratory installations and is validated regularly. After being autoclaved, the waste was stored in a sealed bin, with biohazard symbols displayed, until removal by a registered waste contractor for disposal.
- Sharp material was disposed of in cin-bins, which are removed by a registered waste contractor for disposal.

Safe storage procedures

- During the experimental phase of the project, the organisms were be stored inside a freezer of a level 1 biosecurity laboratory inside the UANL's School of Biological Sciences facilities.
- For long term storage, the culture was frozen and left at -80 Celsius for storage purposes.

SPECIAL THANKS

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