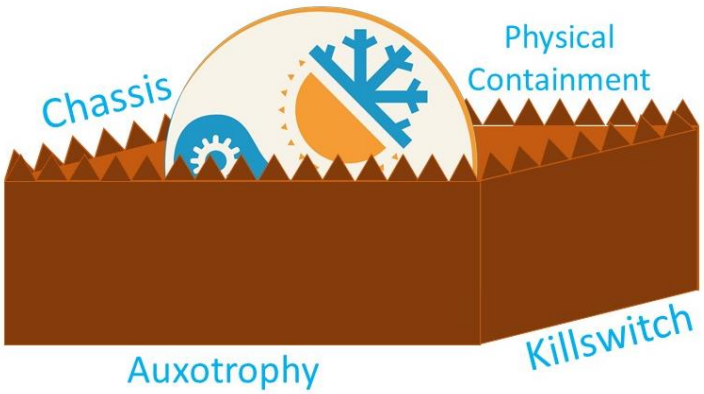
Biosafety: Killswitch and contamination-limiting diffusion



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This report has been written for the project Softer Shock by the Igem Ionis 2017, but aims at giving a general insight on foliar application for other IGEM teams in the future.

1) On Foliar applications - Mechanisms, parameters and regulation
2) Working with the plant: Analysis of bacterial flora and chassis selection
3) Protein choice: Mechanisms of action at low and high temperatures and secretion/size
4) **Biosafety: Killswitch and contamination-limiting diffusion**
5) Potential impact of our product on health (toxicity) and wine taste

Summary:

Introduction:

The development of synthetic biology has raised numerous questions and considerations about **ethic** and **safety**. Indeed, even though artificial engineered systems are **not supposed to harm humans** and more generally, **ecosystem**s in either way (as with toxin encoding gene or antibiotic resistance gene) it is widely known that it needs to be **controlled**. Indeed, this is due to the possible **escape** of organism or its genes from their intended habitats. The genetically modified organisms have been engineered mainly to **help** humans and they possess **new genes**, **new machineries** that enable them to **produce and metabolise new compounds or catalyse reactions**. However, these machineries **need energy** and are not favourable for microorganisms, therefore it seems that they **can not compete** in the environment.

However, there is **still a risk**, and they still could have **negative environmental impact**. These impacts could be **direct**, as they could **compete with other species** and **increase antibiotic resistance**, but also **indirect** as they could also **imbalance** native species interactions.

It is then obvious that **biosafety should be considered in the represented projects** during the IGEM competition, and even more obvious for a project such as Softer Shock, in which the genetically modified microorganisms are aimed to be **spread in a limited environmental area**.

The goal of this report is to give a brief **overview** of biosafety and how it has been considered in **our framework**. In our particular case, biosafety has been thought for all the **environments other than vineyards** our device could reach, and particularly as it has been designed to be the **less harmful possible on vineyards**. The impacts of our project on vineyard are discussed in the other the IGEM Ionis 2017 reports (see report 2 “Working with the plant: Analysis of bacterial flora and chassis selection).

1) Definition and biosafety concepts

Biosafety is originally defined by the **BMBL** (*Biosafety in Microbiological and Biomedical Laboratories*), which is well considered for biosafety practice and policy in United States, as : "The discipline addressing the safe handling and containment of infectious microorganisms and hazardous biological materials" [1]

In synthetic biology, **biological containment** aims at **avoiding or limiting the spreading** of GMM's traits or their specific genetic material into the environment.
Even though the original definition is about infectious/hazardous biomaterial, which is not supposed to be the case in the project, we will talk here about "biosafety" as it gathers many containment concepts.

Originally, GMOs were designed and produced in laboratories and meant to remain enclosed. The first containment system was **physical**. Indeed, these systems were, and still are, based on the same systems used in laboratories for containment of hazardous biomaterial and depend on the hazard group the microorganism belongs to.
For instance, the containment measure in United Kingdom are summarized in figure 1 and are based on the different hazard group CL-1, CL-2 and CL-3 (see figure 2)

CONTAINMENT MEASURES	**CL-1**	**CL-2**	**CL-3**
Room			
Isolated Laboratory suite	not required	not required	required
Laboratory sealable for fumigation	not required	not required	required
An observation window or alternative so that occupants can be seen	yes / no (based on risk assessment)	yes / no (based on risk assessment)	required
HEPA filtered lab extract and input air	not required	not required	required for extract only
Entry to lab via air lock	not required	not required	yes / no (based on risk assessment)
Impervious/easy to clean surfaces	required for bench	required for bench	required for bench and floor
Negative pressure relative to the pressure of the immediate surroundings	not required	yes / no (based on risk assessment)	required
Equipment			
Laboratory to contain its own equipment	not required	not required	required

Use of microbiological safety cabinet/enclosure	not required	yes / no (based on risk assessment)	required
Autoclave	required on site	required in building	required in lab suite
System of work			
Access restricted to authorised personnel only	not required	required	required
Specific measures to control aerosol dissemination	not required	required to minimise	required to prevent
Shower	not required	not required	yes / no (based on risk assessment)
Protective clothing	required	required	required
Gloves	not required	yes / no (based on risk assessment)	required
Efficient control of disease vectors (eg for rodents and insects) which could disseminate GMMs	yes / no (based on risk assessment)	required	required
Specified disinfection procedures in place	yes / no (based on risk assessment)	required	required
Written records of staff training	not required	yes / no (based on risk assessment)	required
Safe storage of GMMs	yes / no (based on risk assessment)	required	required

Figure 1: GMM containment measures applied in University of Bristol, UK [2]

Information box: Hazard group definitions When classifying a biological agent it should be assigned to one of the following groups according to its level of risk of infection to humans.	
Group 1	Unlikely to cause human disease.
Group 2	Can cause human disease and may be a hazard to employees; it is unlikely to spread to the community and there is usually effective prophylaxis or treatment available.
Group 3	Can cause severe human disease and may be a serious hazard to employees; it may spread to the community, but there is usually effective prophylaxis or treatment available.
Group 4	Causes severe human disease and is a serious hazard to employees; it is likely to spread to the community and there is usually no effective prophylaxis or treatment available.

Figure 2: Hazard group definition by Health and Safety Executive, UK [3]

Moreover, laboratories generally need a **certification to use GMO**. In france, a laboratory which wants to modify organisms needs to be **accepted by the Government**, based on the assessment by the "**Haut Conseil des Biotechnologies**" ("High Council of Biotechnologies") which checks the used material for containment, for instance[4].

However, there is an **increase in the use of GMO** in large-scale or open environment due to their use for bioremediation, bioenergy or agriculture, despite the need of **new containment methods** other than physical in order to to protect the environment[5].
These systems now consist of modifying microorganisms (in our case) as we can **set up the conditions of their deaths and avoid unintentional spreading**.

Torres and colleagues gathered these containment systems under three routes:

- Modification of biological production manufacturing
- Modification of individual components
- Modification of biological processes. (Figure 3)

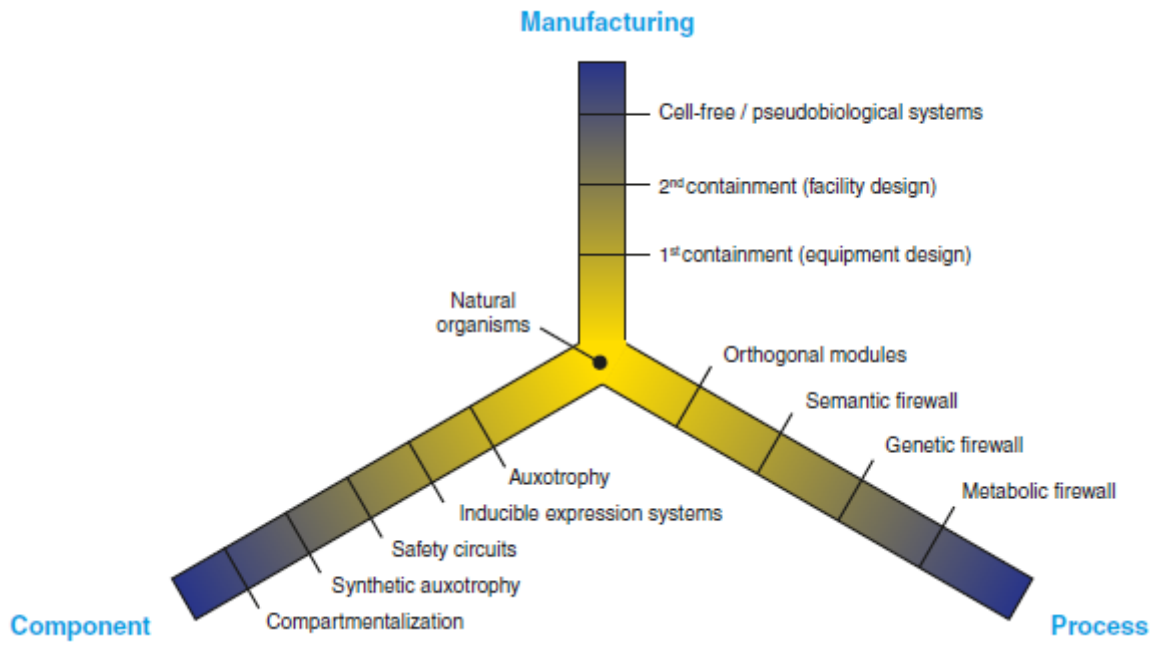

Figure 3: Route to biological containment (Yellow to blue correspond to easy to difficult to develop) [6]

Above these examples, the three main systems that are the most established are:

- Auxotrophy
- Inducible expression systems
- Safety circuits

2) Main established biocontainment strategies

a) Auxotrophy

Auxotrophy is defined as "**requiring a specific growth substance** beyond the minimum required for normal metabolism and reproduction by the parental or wild-type strain".

Basically, **not providing growth medium anymore** to a strain is a kind of **natural auxotrophic containment**. They are not able to produce the compound they need, and only external supply allow them to grow. It is already widely used in laboratories and some of them have developed strains that **require a vital compound in great amount to survive** as for instance, the χ1776 *E. coli* strain which were developed at the early stage of cloning and which **lacked functional aspartate semialdehyde deshydrogenase, L-delta-1-tetrahydrodipicolinate synthetase and thymidylate synthetase**. Therefore, this strain needed an external supply of diaminopimelic acid and thymine/thymidine[7]. However, for our application **it is not enough** for many reasons: the external compound could **easily be found in the environment**, **mutations** can happen and circumvent the modified gene, **other compound can replace the lack** of natural one, and finally the organisms seem to have **limited growth** even with great amount of external supply compared to non- modified ones.

b) Inducible expression system

This is the **least effective** containment, and is among the **basic tool** of synthetic biology. It relies on the gene expression platform, as **operon or specific promoters** are **up/downregulated** by the presence of some elements such as sugars, substituted benzenes, cyclohexanone-related compound, propionate, alkanes, peptides etc.
Well known elements are the **tetracycline promoter** with anhydrotetracycline, or the **lactose operon**, which shut down the transcription of related genes in absence of lactose.
In our project, we use another class of regulated promoter, along with the ones regulated by light for instance, the promoter regulated by **physical trigger**. This is the case with the **pL promoter**, which is **upregulated above a certain temperature**. It is very useful for our application but can not be employed as for containment. Indeed, the idea is that with not any trigger outside the lab, the engineered organism does not display the trait and loose its benefit. However, the organism would **still be there outside**, with modified genetic material.

c) Toxin/antitoxin killswitch strategy

With a killswitch, synthetic organisms depend on specific and synthetic environmental compounds to function correctly. This is a **passive pathway**.
Also, researchers have engineered synthetic organisms that **automatically kill themselves** outside of their specific and selected environments: it thus becomes an **active pathway**. This technique has been designed by observing nature and because a lot of bacteria contain a

specific toxin/anti-toxin system that induces a self-balance. If the anti-toxin activity does not exist anymore due to a loss of the plasmid carrying the anti-toxin, the toxin then kills the cell.

For the first synthetic and self-killing organisms, this toxin system was employed in simple kill switches, where the exogenously supplied **small molecules were repressing the toxin expression**. Without these effectors, the cell would express the toxin and so killing itself.
As an example, in a study by Andersson and colleagues, they expressed the membrane-depolarizing toxin hok gene under the trp tryptophan repressible promoter in E. coli; in the case of the absence of a high concentration of tryptophan in media, the organisms would express hok, and thus kill themselves.

Later, several researchers used **LacI-based** inverters to construct some killswitches that were **activated by the absence/presence of a larger amount of synthetic molecules**, such as the kill switch in P. putida, where the 3-methylbenzoate-activated TOL promoter induces production of LacI, which in turn repressed the toxin gef. An absence of 3-methylbenzoate turns off LacI expression and thus gef repression, resulting in gef expression-mediated cell death.
Genes that sequester key metabolites make the cells starve: they can be used like an alternative to toxins, like Cantor and colleagues who developed a switch where the killing condition is the overexpression of streptavidin, which is able binds and sequesters biotin, its key metabolite. It then leads to cell death.

These single-component kill switches are **usually quite strong**, but they are also quite **subject to failure** because of different point **mutations**, inactivating the killing mechanism.
Moreover, recent advancements have allowed the development of **multilayered kill** switches that are **more robust** and even **more dependent** on artificial environments.
Indeed, Collins and colleagues developed different architectures of kill switches, consisting of **networks of component that interact together to reinforce the killing state** in the absence of a strong, highly specific and non- killing environmental signal. It provides a **backup** in case one component is mutated or otherwise non-functional.

Recently, the **“DEADMAN” switch** has been developed. It uses a **stable regulator** with different **reinforcing feedback loops**, that activate the expression of the toxin but also the degradation of an essential cell protein in the absence of a specific effector. The **“PASSCODE” switch** requires the **presence and absence of a specific combination of synthetic effectors** (which is an AND/NOT gate) to repress toxin expression. Although they reported escape frequencies do not explicitly improve upon those of previously reported kill switches (that is 30) or addiction strategies, these architectures presumably would show improved stability.

Here are different types of toxin/anti-toxin killswitches, with their different strengths and weaknesses in case of a willing of use.

System name	Brief description	Strengths (+) and weaknesses (−) for use	References
Toxin–antitoxin systems			
Type I e.g. Hok/*sok*, Fst/RNAII, TxpA/*ratA*	Type I toxin–antitoxin systems consist of a membrane-active protein toxin whose expression is repressed by a short RNA antitoxin; the *E. coli* Hok (6.1 kDa)/*sok* (65 nt) system is the best characterized of these, originating from the *parB* locus of *E. coli* plasmid R1; Fst (3.7 kDa)/RNAII (66 nt) is a Gram-positive equivalent from *Enterococcus faecalis* plasmid pAD1 and TxpA (6.7 kDa)/*ratA* (222 nt) is a homologue found on the *B. subtilis* chromosome	+ Low metabolic load due to post-transcriptional regulation − Type I systems appear to be specific for Gram-negative or -positive cells + Hok is a well-described post-segregation killing system for Gram-negative bacteria, several homologues available (e.g. HokC [*gef* gene]) − Hok-resistant *E. coli* persisters can result from overexpression of Hok − TxpA will require genomic knockout for use in *B. subtilis*	Gerdes *et al.* (1997), Gerdes & Wagner (2007), Brantl (2012), Weaver (2012), Durand *et al.* (2012)
Type II (RNase) e.g. Kid/Kis, Txe/Axe, MazF/MazE, RelE/RelB, HicA/HicB	The majority of described type II toxin–antitoxin systems consist of an endoribonuclease paired with an inhibitor antitoxin protein; Kid (14.8 kDa)/Kis (9.3 kDa) is from the *parD* locus of *E. coli* plasmid R1 and Txe (10.2 kDa)/Axe (13.6 kDa) is from the *axetxe* locus of *Enterococcus faecium* plasmid pRUM; further well-characterized examples that are native to the *E. coli* genome include MazF (12.1 kDa)/MazE (9.4 kDa) from the *rel* operon, RelE (11.2 kDa)/RelB (9.1 kDa) from the *relBE* locus and HicA (6.8 kDa)/HicB (15.2 kDa) from the *hicAB* locus	+ Type II endoribonuclease toxin–antitoxin systems are well described, with many homologues available in diverse bacteria − Type II endoribonucleases are bacteriostatic rather than bactericidal + Kid, Txe, MazF and RelE and HicA are broadly active in both Gram-positives and -negatives ± Kid, MazF and RelE are known to be toxic when expressed in eukaryotes − Txe/Axe is not well characterized − MazF, RelE and HicA all require genomic knockout for use in *E. coli*	Pedersen *et al.* (2002), Grady & Hayes (2003), Pedersen *et al.* (2003), Kamphuis *et al.* (2007), Neubauer *et al.* (2009), Diago-Navarro *et al.* (2010), Halvorsen *et al.* (2011)
Type II (gyrase inhibitor) e.g. CcdB/CcdA	CcdB (11.7 kDa) poisons DNA gyrase to inhibit replication, while CcdA (8.4 kDa) acts as the labile antitoxin; sourced from the *ccd* locus of *E. coli* plasmid F	+ Well described, analogues available (e.g. ParE/ParD from plasmid R2K) − Only active in Enterobacteriacae − Intellectual property is claimed on this system	Bernard & Couturier (1992), Bernard *et al.* (1994), Jiang *et al.* (2002)
Type II (translation inhibitor) e.g. Doc/Phd	Doc (13.6 kDa) stabilizes bound mRNA at the 30S ribosomal subunit to block translation; this is relieved by Phd (8.1 kDa); sourced from the prophage plasmid of bacteriophage P1	− Bacteriostatic rather than bactericidal ± Unknown if Doc is toxic in eukaryotes, but expected to be given that the site of action is the same as the aminoglycoside hygromycin B	Brodersen *et al.* (2000), Liu *et al.* (2008)
Type II (other) e.g. Zeta/Epsilon	Zeta (32.4 kDa) phosphorylates UDP-GlcNAc, preventing its use by MurA in cell wall synthesis; epsilon (10.7 kDa) acts as the antitoxin; sourced from *Streptococcus pyogenes* plasmid pSM19035	+ Active in Gram-positives and -negatives − Not as toxic to Gram-negatives as Gram-positives ± Only mildly toxic in eukaryotes (e.g. *Saccharomyces cerevisiae*)	Zielenkiewicz & Ceglowski (2005), Mutschler & Meinhart (2011)
Type III e.g. ToxN/ToxI	ToxN (19.7 kDa) is an endoribonuclease that is directly inhibited by pseudoknots encoded at the 5′ end of its transcript (ToxI region; 5.5 repeats of 36 nt); sourced from *Pectobacterium atrosepticum* plasmid pECA1039	+ Broad presence in both Gram-negatives and Gram-positives + The *cis*-encoded RNA antitoxin reduces metabolic load on cell − Bacteriostatic rather than bactericidal	Fineran *et al.* (2009), Blower *et al.* (2011, 2012)

Figure 4 : Strengths and weaknesses for each type of device as a biosafety mechanism[6bis]

3) Development of new complex systems

Even though these systems are **theoretically** well designed, it appears that **single layer** containment is **not enough** to ensure very low escape frequencies as we mentionned above. Indeed, the gene responsible of death (for instance a toxin) can be **bypassed mainly through mutation** as it requires a low evolutionary cost.

To overcome this issue, it has been made possible to design **more complex systems**, either by affecting **cellular processes** (semantic biocontainment) or adding **multi-layered systems** for instance.

Here we will just give an overview of some example which have been developed in this aim and that we encountered during the project.

a) Synthetic and advanced auxotrophy, a trophic biocontainment

As mentioned above, auxotrophism is supposed to offer a way to **limit expansion of organisms** into the area supplying the metabolite for survival. However, this technique has a huge drawback: most of the natural metabolites an organism could need are also used by other organisms and **very likely to be found** in nature.
However, a way to solve this problem is to engineer organisms to be **dependant on a synthetic metabolite** or at least **not found in nature**.

For instance, Lopez and Anderson engineered *E. Coli* BL21 as three of its essential genes (tyrS, metG and pheS) are **dependant on the presence of benzothiazole**. They used the evolution platform SLiDE (Synthetic auxotrophs based on Ligand-Dependent Essential genes) to find the strain. Basically, they **modified the candidate genes** by random or targeted **mutagenesis**, and did **dual selection** using chemical complementation and penicillin. Their mutant had **no escape detection** (below 3x10E-11) but reached 10E-6 after some days[7bis].

In order to decrease the escape frequency by developing another strategy compatible for multi-layered containment, Ryiuichi Hirota and colleagues developed an *E. coli* strain so that it expresses **phosphite deshydrogenase** and a **P transport system,** which is only able to take up **phosphite and hypophosphite** but not phosphate. Plus, all the **endogenous P transporters have been disrupted**. With this construct, phosphite and hypophosphite are the only vital source of P, and it has been found that they are rarely found in environment, and not enough to allow them to survive. This strategy leads them to the **lowest escape frequency** known at their time (Article received November 1st 2016), which was no escape for at least 21 days with a detection limit of 1.94x10E-13 per colony[8].

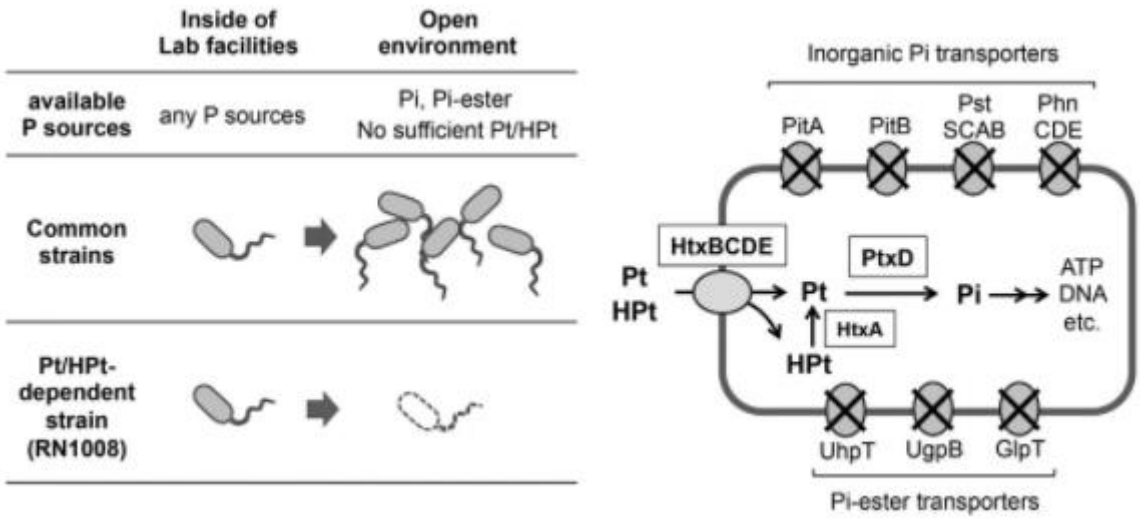

Figure 5: Creation of engineered dependency on phosphite/hypophosphite

However, the containment **solely based** on auxotrophy suffers from the drawback that **mutations can happen** and lead to mutant that **no longer requires the external compound**. It is then possible, at least to try, to avoid the problem by **combining** auxotrophic containment with semantic containment.

b) Semantic containment and anti-Horizontal Gene Transfer strategy

As stated above, the auxotrophic containment could be circumvented by mutations but it also does **not address the spreading of genetic information**. Indeed, **horizontal gene transfer** can occur between bacteria but also, even if a killswitch mechanism is used for instance, the genetic material of dead individuals **become free** in environment and could (even though not all organisms are naturally competent) be taken by wild type bacteria.
It may not be the principal issue in a laboratory but in our project, this aspect is really relevant and is something we cannot let aside.

The **semantic containment**, which may be harder to set up, provides an efficient way to avoid this problem by designing what is called **genetically recoded organism** (GRO). In fact, semantic containment aims at **modifying an organism's the genetic code** by **reassigning the codons** with another specific essential/canonical amino acid.
The genetic code is almost universal for all organisms, and is composed of **64 triplets** (codons) of nucleotides (A, U, C, G) that determine which of the **20 standard amino acid** (Plus 2 derived from stop codon) will be added by the ribosome during protein synthesis. 3 of theses codons are punctuation (stop codon). When mRNA is read, **Synthetase** attaches an amino acid to specific receptors and tRNA which becomes **aminoacyl-tRNA** (aa-tRNA) and then **attaches the amino acid to the synthesizing protein**. The attached amino acid depends on the read codon[9].
With semantic containment, codons can be artificially reassigned in three ways: **sense to sense** codon, **sense to stop codon** or **stop to sense codon** (Figure 6).
For example, by mutating synthetase or tRNA, a linkage between an amino acid with a tRNA should not occur. Therefore, the same DNA would not be read (and give the same protein) the same by the recoded bacteria and the wild type one[6,9].

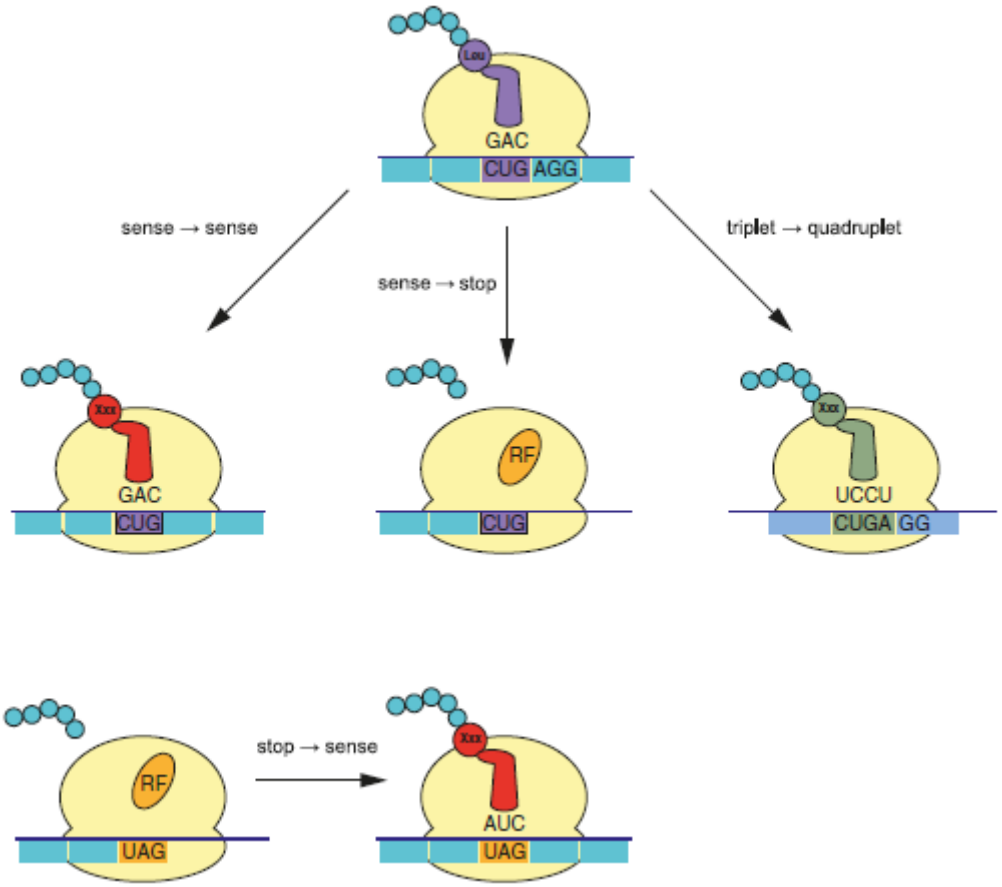

Figure 6: Different ways to semantic biocontainment [6]

Recoded organisms prove a **good efficiency in containment**, regarding to escape frequencies (even more when combined with auxotrophic containment, see below) but is **not simple to set up**. Indeed, it needs **tRNA/synthetase** modification and it is also seeking for **relevant candidate protein gene using computers and modelling**. However, some other ways to contain the spreading of genetic material exist.

One of them is to use a **killswitch system based on DNase**. Torres and colleagues developed a mutant which carries on the plasmid a coding gene for **EcoRI**, a **DNase** (EcoRIR) and a **colicin E3** (ColE3) which cleaves specifically 16S rRNA. Also, they chose E. Coli K-12 strains which constitutively **express immE3, the immunity E3 protein** (which inhibits RNase activity) and add a EcoR1 methyltransferase (EcoRIM) coding gene into the chromosomal DNA using transposons.
Therefore, there is a couple **EcoRIR/ColE3** into the plasmid, which acts as a toxin by **targeting both DNA and RNA**, and there is a couple of their anti-toxin immE3/EcoRIM encoding into the genomic DNA.
Basically, the idea is that the plasmid can only be carried by these modified strains as there is a balance between the production of toxin, and their inactivation by anti-toxin, but in the case where a horizontal gene transfer of plasmid occurs, the wild type bacteria would **die as they do not carry the anti-toxin genes**[10].

c) Combining the idea, an example of multi-layered strategy

A lot of biocontainment strategies have emerged since the beginning of synthetic biology, offering low escape frequencies. However, most of authors agree to say that **only one system is not enough** and that systems that can combine **several pathways** are needed; even though for now, multi-layered system show additive results rather than synergistic.
A well-known instance of combining strategies is using **both semantic and synthetic auxotrophic** containment.
Indeed, the semantic protection **avoids the genetic information to be read** by other organisms, and the synthetic auxotrophy traps the organisms itself.

For example, Lajoie and colleagues worked on *Escherichia coli* and make it **dependant on L-4,4'-biphenylalanine (BFA)** by identifying and engineering specific sites in six essential proteins, so they could **replace one amino acid with the non- essential amino acid BFA**.
The idea is to **reassign a stop codon** (here UAG, which is a codon with few occurrences in *E. coli* genome) by **suppressing the RF1** (factor that stops the translation at the UAG stop codon) and assign an orthogonal translation system composed of aminoacyl-tRNA synthetase: tRNA to UAG, in order to allow the **use of a synthetic amino acid at this position**. Then, all the initial UAG are replaced by another stop codon and occurrences of the new UAG (coding for the synthetic amino acid) are inserted into the protein gene of interest[9]. (Figure 7) For this, they need to **identify site candidate** where the amino acid would be essential without being disruptive. In this aim, modelling prediction is essential. It is also how Rovner and colleagues inserted different synthetic amino acid into 22 essential genes of *E. coli*[10] (Figure 8).

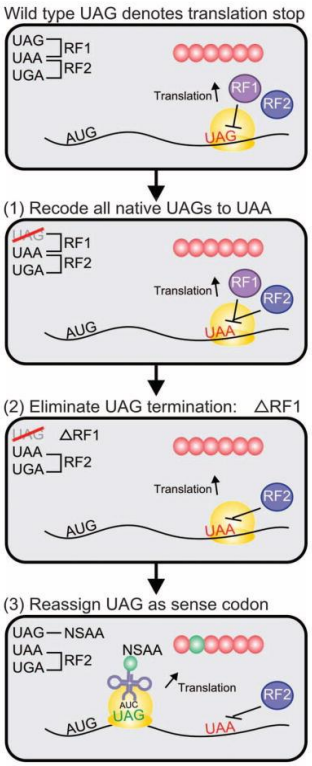

Figure 7: Engineering GRO to depend on sAA

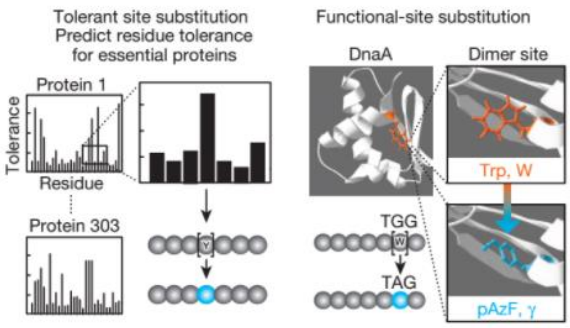

Figure 8: Modelling prediction for amino acid substitution[10]

Other methods have emerged more recently but this one has been cited a lot and remains with **no detectable escape frequency during 20 days** in liquid media and **7 days** in solid media after culturing 10E11 cells.

4) In our project: Softer Shock

As stated above, our project relies on the **spreading of our engineered bacteria** onto the vineyard, therefore we are aware that ensuring the containment of biomaterial as bacteria inside such a wide area is a tough challenge. However, we think that, even if we did not find the "perfect" strategy, we might provide some leads for biocontainment in this kind of open environment.

Here we will share and discuss our different strategies through the development of the project, based on the bibliography mentioned above. (Author's note: The strategies were developed in the same time bibliography was in progress. The papers mentioned above are not listed by time of discovery. Therefore, we did not have all of these informations at the time we thought of different strategies.)

a) First strategies

At the very beginning, we heard about the **"Passcode" and "Deadman"** systems that had been set up by the **MIT**[11] and quickly wanted to work on a killswitch system in order to contain our microorganism.

We firstly sought for a **toxin** that could suit. As we maybe wanted to work with biopolymers, the choice of **Protegrin-1**, which is known to **bind to lipopolysaccharides** and to **insert into membrane**, then inducing poration of gram-negative bacteria, appeared to be good. Indeed, slight induction of protegrin-1 would be enough to let the biopolymer (or protein) be secreted without signal peptides, and a **greater induction would disrupt the membrane enough** to let the bacteria die.

Then the principal issue we faced was: **when** are they supposed to die and **how** could we set up this mechanism?

As we did not want to use any ligand-inducible promoters and that none of the inducible promoters with for instance light suited with the project, we thought of **inducing the protegrin-1 activity at the same time than our protein of interest**, after a heat/cold shock in a mild manner.

We also took the problem of **horizontal gene transfer** very seriously, and we wanted to integrate a **DNase/Anti-DNase** system as the EcoRIR/EcoRIM cited above. (Figure 9)

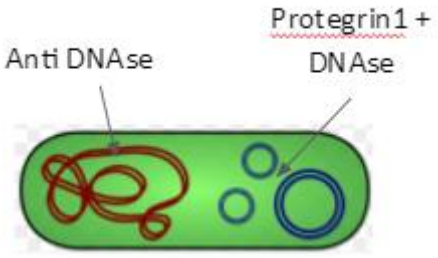

Figure 9: The first system in a nutshell

However, it contains many drawbacks and issues. Indeed, if we look at the different possibilities we have:

IF heat/cold shock:

Protegrin-1 is transcripted at the same time than our protein of interest

OK

BUT it makes it usable only one time, therefore it is not convenient to winegrower

ELIF horizontal DNA transfer:

DNAse coded into plasmid is free to act as wild type bacteria don't have antiDNAse

OK

Else:

No activation killswitch. They should die as it is not an « advantage » and is energy consuming

NOT OK (Too much hazardous, random)

Moreover, we finally **did not find an easy way to be sure** our engineered bacteria would be able to synthesise enough protein of interest before dying. Many ideas were suggested in order to circumvent the problems. We will not discuss about them here, but finally this kind of induction has been let away.

So, we then worked on specificity. Basically, the idea was to ensure that our bacteria would really **need the grapevines** and that it would **stay on it** in order to survive. In this aim, we both investigated a **chassis** for our construct which would specifically live on grapevines, and we sought for a **promoter which would be inducible** by a specific compound of the grapevines.

The chassis has been discussed in the the report *Working with the plant: Analysis of bacterial flora and chassis selection*.

The most specific compound which has been found for grapevines is the **pectin** and its derivatives, such as **polygalacturonic acid**.

There exists a promoter (PGU1) from *Saccharomyces* which is upregulated first through a **Kss1 MAPK** (Saccharomyces MAPK) and then **upregulated by galacturonic acid**. It has been found that *E. coli* is able to express some genes from a yeast as Saccharomyces[12], therefore based on the hypothesis that this promoter would work with *E. coli*, it should only be upregulated by the presence of galacturonic acid. The grapes, as other soft fruits, are poor in pectin as it underwent more reaction with pectinase (which break down pectin) than fruits as citrus or oranges, so they should have more galacturonic acid residue.

This promoter could be used to **regulate the transcription of an anti-toxin**. In the absence of galacturonic acid, which would be in the case the bacteria escape the vineyard, **the anti-toxin is no longer produced and the toxin kills the escapee[13]** (Figure 10).

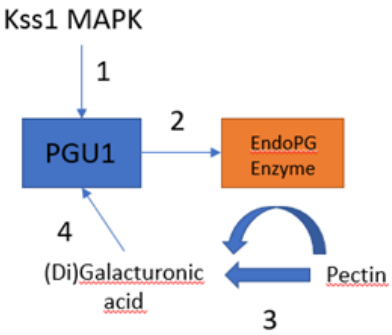

Figure 10: PGU1 mechanisms

Again, this system relies on many random factors as if the **promoter would work on *E. coli*** or if there would be **enough galacturonic acid** on vine grapes but also it seems that **PGU1 is autoregulated with galacturonic acid** and the main problem is that pectin and galacturonic acid is not only found on fruits but also dicotyledon. Therefore, as we had not enough informations, only the idea of chassis remained (see further or the report "Working with the plant")

Finally, as we could not find a **unique strategy** able to ensure a "satisfying" biocontainment whatever the scenario which could occur, we thought that only **multilayer containment** could fit. We needed **different strategies** able to handle the different cases of escape and ultimately, which could have additive impact upon each other. This where the idea of the "**four walls"** came up from!

b) The four walls: Chassis/Physical/Auxotrophy/Direct Killswitch

- **Synthetic Auxotrophy**

At the beginning, we did not want to use an **external supply** of product, in order for instance to trigger killswitch or to make them survive (natural auxotrophy) as it could **disrupt natural balance in the wide phyllosphere** but also the plants themselves. Indeed, using an inducer as IPTG or lactose could **trigger some wild type strains** for instance. But we thought that using **synthetic auxotrophy** might work. As the used amino acid is not naturally present in nature and is a biological product, it should have **no interaction** with the environment.

For practical reasons, the chosen amino acid at the required concentration needs to be cheap. And so, **the L-4,4'-biphenylalanine** used by Lajoie and colleagues9 could be used. (Table 1) Indeed, this solution does not avoid the need to spray on vineyard and **should not exceed too much the price** that farmers currently pay to apply water.

NSAA	Vendor	Name at vendor	CAS#	MW	Cat# for 1g	Price of 1g	Optimal conc. (mM)	Cost per liter of culture
pAcF	peptech	L-4-Acetylphenylalanine	122555-04-8	207.23	AL624-1	$500.00	1.0	$103.62
pAzF	Bachem	H-4-Azido-Phe-OH	33173-53-4	206.2	F-3075.0001	$285.00	5.0	$293.84
pCNF	peptech	L-4-Cyanophenylalanine	167479-78-9	190.2	AL240-1	$150.00	1.0	$28.53
bpa	peptech	L-4-Benzoylphenylalanine	104504-45-2	269.3	AL660-1	$100.00	1.0	$26.93
napA	peptech	L-2-Naphthylalanine	58438-03-2	215.25	AL121-1	$80.00	1.0	$17.22
bipA	peptech	L-4,4'-Biphenylalanine	155760-02-4	241.29	AL506-1	$150.00	0.1	$3.62
pIF	peptech	L-4-Iodophenylalanine	24250-85-9	291.09	AL261-1	$40.00	1.0	$11.64
bipyA	Asis Chem	Bipyridylalanine	custom synthesis	245.282	(25 g price)	$10,000/25g	1.0	$98.11

Table 1: Cost per liter of culture for different synthetic amino acid

Using **moderate sAA dependant strain**, and allowing them a **reserve of sAA in microbeads** should make them survive some time making it not too inconvenient and too expensive.
This idea is the **main containment** to ensure that there will not be modified microorganism running into the wild after a certain time.

- **Killswitch and Horizontal gene transfer avoidance**

Even though the synthetic auxotrophy relies on semantic containment and **should avoid bacteria spreading** and make the DNA not readable for other organisms, we thought about **adding another system** specifically against horizontal gene transfer. Indeed, our bacteria could “give” their DNA to other bacteria through this phenomenon. Plus, competent bacteria are rare but do exist in nature and **can integrate free DNA from dead modified organism**. Thus, a **DNase/anti-DNase** (and/or RNase) as EcoRIR/EcoRIM should be implemented to avoid any horizontal gene transfer. The only drawback of this method is that it **may kill organisms** that acquire the plasmid.

Then a killswitch could be set up for **harvesting**. Indeed, in order to ensure that no remaining organisms will be on the vine grapes, a Protegrin-1 gene could be inserted under the control of an **lactose operon**. When the harvest is done, the vines grapes could be **washed a first time with a lactose solution, and a second time to remove dead bacteria**. (Figure 11)

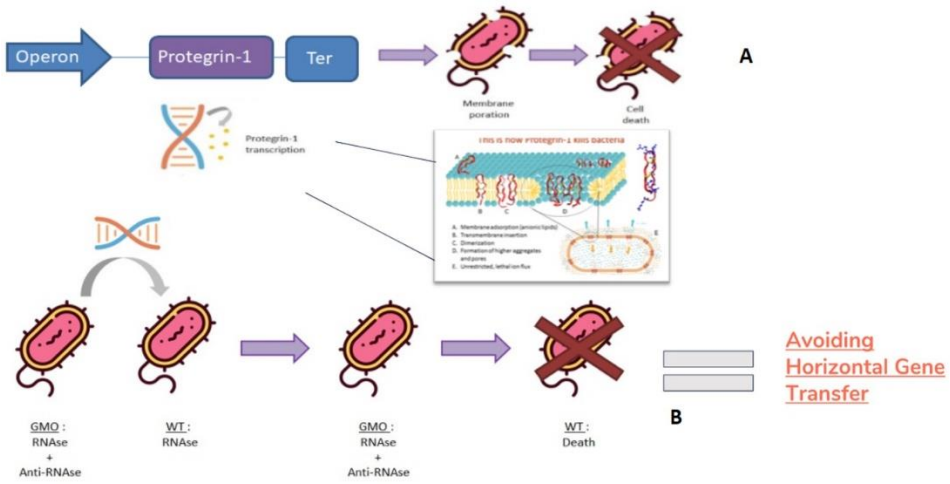

Figure 11: A) Killswitch with Protegrin-1 B) Avoiding Horizontal Gene transfer

- **Limiting contamination and diffusion through physical ways.**

Biosafety is not only about killswitches and the use of metabolic pathways. In Softer Shock, the physical containment our our microorganism is of course directly compromised by the fact that we would release it in the environment on grapevines. Once the application delivered, there is **a risk of spray drifting** that would bring the product out of the desired zone of application. There is also a risk that our microorganism ends up in **soil** and eventually infiltrate it as well as **water** that passes closely. To limitate the drifting and deviation of Softer Shock, we are going to rely on two technologies that will form another wall of safety for our project.

Adjuvants:

These compounds have been widely treated in **the foliar application report** so you might want to check it out. In brief, adjuvants are **added substances** to the application to enhance some of its properties. Amongst these properties, some are crucial to us:

-**Drift retardant**, meaning the adjuvant reduces spray drifting. Important to note, during our meeting with Syngenta, they mentioned that this technology was not very relevant so it might not be as meaningful for our project.
-**Bounce and shatter minimiser**: as the spray can indeed bounce on target and end up in environments like soil, this type of adjuvant could be very useful.
-**Sticker and retention aid**: Increases the solution retention and resistance to rainfalls. This also very interesting for us to minimise the departure of our organism from target after rainfalls. On the other hand, Syngenta experts indictaed that these adjuvants worked very well[14].

These three properties are the interesting ones for our project when we refer to biosafety. If you want more information on adjuvants, look at the report "**Folliar Application**"!

Adjuvants can hence, from the very beginning and the formulation of our product, induce a soft containment of Softer Shock in the natural environment during and after application. We are aware that it is **not sufficient** at all, but we want to **maximise the chances**. Another physical biosafety process will be added to the project, called harvesting panels.

Tunnel sprayer:

Originally used to protect bark problems of vineyard in the years 1990's, these technologies have been let down after the ban of the sodium arsenite. Since then, **few farmers have decided to maintain their use for products** such as pesticides in vineyard protection.
The tunnel sprayers are devices based on a "face to face" model in which each of the product dispenser face each other.

Here are some pictures :

BERTONI Arcobaleno | FRIULI Drift Recovery | WEBER NC1000 UEZ QU

Models of tunnel sprayers by different manufacturers[15]

And more ![16]

Now you can picture yourself what we meant by face to face! This technology is really important because as the two panels surround the vineyard, just like this:

Two panels surrounding a grapevine tree [16]

The product that is sprayed on one side and doesn't end up on the plant is **harvested by the panel on the other side** instead of being released in the environment and the soil or closeby water! Another really nice quality of such system is the fact that it permits the **deposit of the spray on both surfaces of leaves** (abaxial and adaxial)[17].

Panels are divided into two technologies:

-**The pneumatic technology**, in which the spray is applied under the form of very fine droplets. This one, even if it still permits to limit the loss of product, doesn't impact the spray drifting that we want to avoid absolutely, hence we are not going to focus on this technology.

-**The airblast sprayer technology** however, permits, through the use of powerful pumps that pressurize the product and air-injecting nozzles that guarantee a bigger droplet size, to apply the product efficiently and limit the drifting to the maximum. Different nozzle types exist but we are not going to go further into details.

The air blast tunnel sprayer is hence the technology we will choose to apply Softer Shock, it will guarantee economic advantages as well as reinforce the biocontainment of our microorganism.

Keep in mind that tunnel sprayers, as good as they seem, also have disadvantages:

-They can **cover only up to two rows of crops** at the same time.
- They require **important cleaning time** and are **hard to maintain**.
-They are usually **slow and hard to manœuvre.**

But recent studies carried on by the French Institute of Wine (IFV) have proven that the **speed of these machines can be increased without affecting their efficiency**, that they tend to

cover most of the canopy and induce around 40% of product reycling each year for an average vineyard[16,17].

These studies show the now **growing interest** of farmers and instititutes for the **tunnel sprayer**. At the dawn of the French government agriculture plan called **French Ecophyto national action Plan**, of which its objective is to reduce the use of plant protection products (PPP) -25% in 2020 and -50% in 2025[17]., the tunnel sprayers are becoming very relevant. For all these reasons we believe the **tunnel sprayer is the right technology to apply Softer Shock and enhance its biosafety.**

The French Wine Institute has shown growing interest in tunnel sprayer 17

- **Chassis selection: integrating metagenomics of the phyllosphere into a species-based and customised biosafety**

Treated in our report "**Working with the plant**", this aspect will be briefly explained here to complete this report. If you want more information on this crucial aspect of Softer Shock, don't hesitate to **read the other report as well!**

As you might have heard or read previously, the **phyllosphere** is the microbial ecosystem of the plant leaves (and at a lesser extent, of plant fruits and barks).[18] This microbial habitat is **populated by a great number of species** of bacteria, eukaryotes, and archaea that try to survive in a harsh environment. Such environment has of course its specificity and studies of the microbial population of the phyllosphere have shown that it was composed of shared species with microbiota of the soil and water, but also of very specific species.[19]

Our goal to induce another biosafety layer to Softer Shock, is to **choose as our chassis and protein producing organism, an organism that is specific to the phyllosphere**.

Our reasoning is the following: if our host, after the application of the spray**, drifts out of the target plant and arrives in the soil or a nearby water source, it will not be adapted** to this unusual environment and will likely die or at least develop very slowly, enough for tests to be carried on and of course for the organism to die because if the synthetic auxotrophs.

Our **choice of microorganism** will be guided through the use of **metagenomics**, science that permits the **sequencing and functional analysis of organisms** present in a **given environmental sample**.[20]

We want to use this science to **analyse the phyllosphere** of a given grapevine from our client as well as the **microbial population of the surrounding environment** such as soil, to have an idea of the species that are present in each environment. We will **integrate** all these informations in what is called a **Geographic Information System** (GIS) (see "Working in the plant" for more details).

We will then **select the chassis according to this integration** and **choose the species that has the maximum desired traits** (see the report "Working with the plant") and guarantee the **highest degree of biosafety possible**.

As **population of microorganisms vary highly** according to **geographic location**, **weather**, **human activity** and many other parameters[21], it is very likely that **a chassis for the application of Softer Shock applied in Bordeaux will not be the same at all as a chassis for the application of Softer Shock in Reims**.
Our chassis selection service will then give rise to a **personalised treatment** that **guarantee efficiency** and, most important of all, **biosafety.**

c) Special attention to antibiotic resistance gene

Antibiotic resistance genes are widely used in synthetic biology as it allows to select only the organisms which have **taken up the plasmid of interest**, and therefore it is used also as a control. These genes are **not an issue as long as the engineered organisms remain into a totally safe and contained environment** but are one of the major concern about the escape of genetically modified organisms in environment. Indeed, in the case where these genes **reach other pathogenic bacteria** it would guarantee **another extra defense** for them, making them even more dangerous. That is why antibiotic resistance genes have to get rid of.
There is a method to extract the entire plasmid from bacteria using **protoplast formation** but in the case we would juste take off the antibiotic resistance gene, we would need tools as **CRISPR-Cas9**.

Conclusion of the report:

Finally, for the biosafety aspect of our project Softer Shock, we chose to create a **four walls fortress**, which means a **multi-layer strategy**.

Our first wall is the **auxotrophy**, and we aim at engineering our bacteria so that they become dependent to a specific component. Here, we chose to make them **depend to a 21st amino acid**, which is not found in nature. Unless it has access to this synthetic amino acid, the **bacterium dies:** it is confined in the area where the amino acid is spread. To do so, we modify a stop codon. The reading frame is changed and in case of a DNA transfer between bacteria, the **DNA is non-readable**.

Our second wall is composed of **a killswitch**, which kills bacteria under certain inputs. It permits the avoidance of a microorganisms spreading. To do so, we chose to use the **protegrin-1**.
The Protegrin-1 causes a **membrane poration** and so the **cell death**. Once its sequence is included in our bacteria genetic code with a lactose operon, it would be activated in the presence **of lactose**, making it easy for the farmers to kill the bacteria after harvesting.
We also thought of adding a **DNAse coding sequence in our plasmid and an "anti-DNAse"** coding sequence in the genomic DNA of our microorganism. If a DNA transfer occurs between a modified and a wild type bacterium, the wild type bacteria which does not contain any anti-DNAse would die.

For our third wall, we are actively looking for the **most adapted chassis** and we already have some tracks of naturally present bacteria on vine leaves and specific to the leaf environment. However, **the perfect chassis does not exist**, as if it extremely specific to the grapevines (so little present) a mass spraying could alter the biodiversity and on the contrary, if it is less specific the safety level would be lowered.

Our last wall is the **physical containment**. We decided to use the **tunnel sprayer** in order to diffuse our product and to add some **adjuvants** to facilitate its use. The tunnel sprayer seems to be a good choice because the product that is sprayed on one side and doesn't end up on the plant is harvested by the panel on the other side. Another point that made use choose this system is that it permits the deposit of the spray on both surfaces of leaves. The adjuvants would be a **drift limitant**, a **bounce and shatter minimiser** and a s**ticker and retention aid**.

The next step to complete our multi-layered system would be to use **modelling** in order to **target the proteins we would use** for the auxotrophy. Indeed, to be dependant of a synthetic amino acid, we need to know what is the best essential protein to use and to modify the codons so that the protein needs this synthetic amino acid to be produced. To do so, we need to **predict where the amino acid would be on the protein sequence**. Modelling is the best way to find the best candidate, as it is way faster but also more precise that bibliography only.

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