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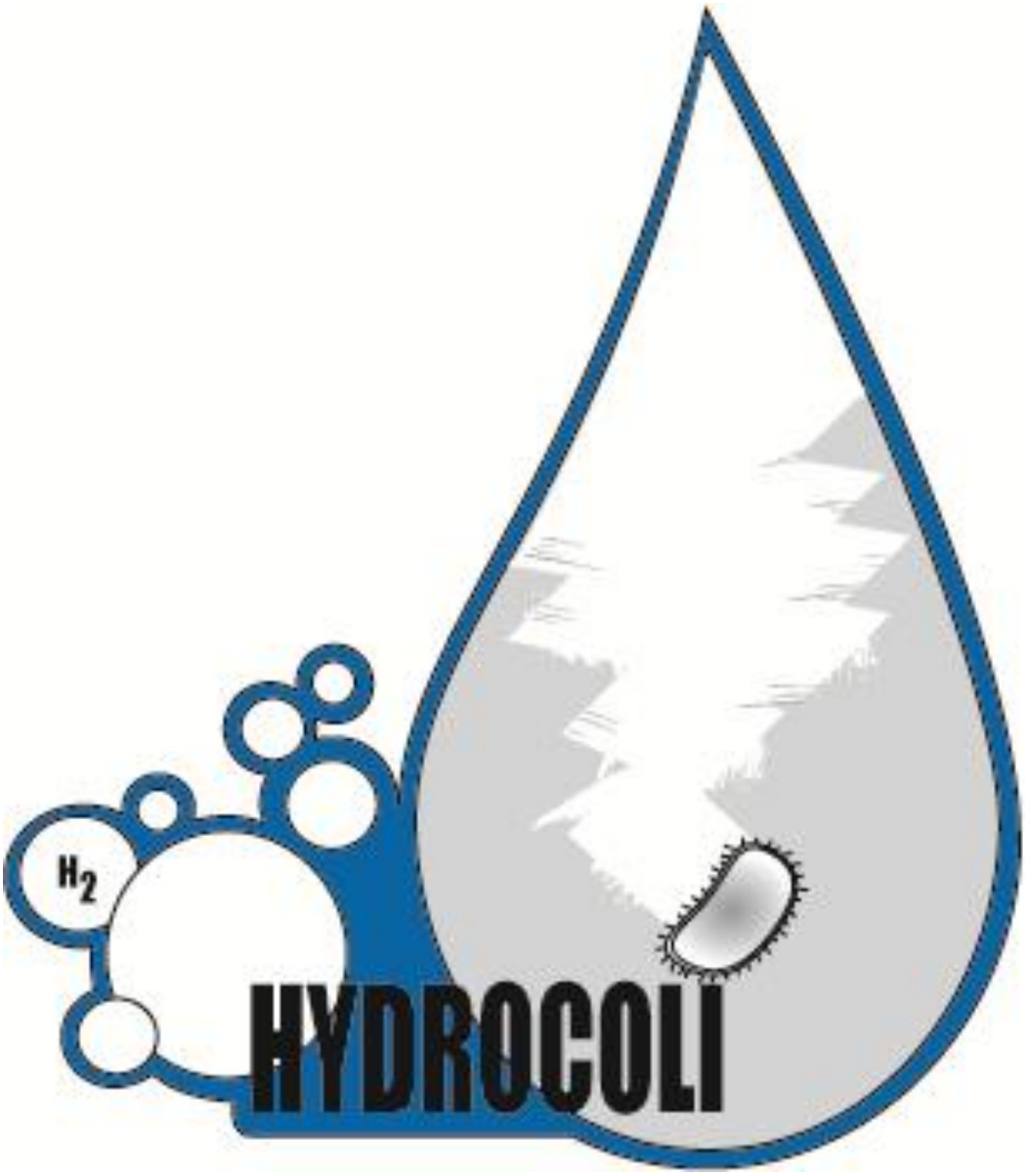

iGEM 2010 TEAM

Université Libre de Bruxelles

Bruxelles, Belgium

ABSTRACT

In this ever more energy-dependent world, where fossil fuel ressources become scarce and raise environmental issues, the search for green energy sources is a growing concern in both civil and scientific communities. In this context, hydrogen turns out to be an interesting alternative.

However, current hydrogen production relies mostly on chemical processes, such as petroleum cracking or water electrolysis. In order to develop greener and more energy-efficient processes, the use of micro-organisms as biocatalysts for hydrogen production has been studied for many years. While no practical application has yet been achieved, nowadays the scientifical and technological advances allow further developments and opportunites in this field.

The actual use of dark fermentation to produce hydrogen attains very low yields, compared to other fermentative biofuel synthesis, e.g. methane or ethanol. We propose to design a genetically engineered E. Coli, with an improved natural hydrogen production pathway, using the organic compounds found in waste waters as substrate. In addition, we will implement various features to enable the strain to perform other tasks related to wastewater treatment, such as signaling metallic contamination, eliminating nitrogen compounds, or hindering hydrogen consumption by methanogenic bacteria. We will also set up a planned death system in order to prevent its proliferation outside the wastewater treatment plant.

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TABLE DES MATIÈRES

I. INTRODUCTION

In this ever more energy-dependent world, where fossil fuel resources become scarce and raise environmental issues, the search for green energy sources is a growing concern in both civil and scientific communities. In this context, hydrogen turns out to be an interesting alternative. Indeed, hydrogen fuel cells do not affect the carbon footprint since their only side effect is a production of water. Although the hydrogen is the most abundant element on the planet, it is quite difficult to produce. Current hydrogen production relies mostly on chemical processes, such as petroleum cracking or water electrolysis, which require a lot of energy.

In order to develop greener and more energy-efficient processes, the use of micro-organisms as biocatalysts for hydrogen production has been studied for many years. A major concern for this approach is the use of dark fermentation, which attains very low yields, compared to other fermentative biofuel synthesis, e.g. methane or ethanol. However, while no industrial application has yet been achieved, scientific and technological advances allow further developments and opportunities in this field.

Turning wastewaters to substrate is convenient in several ways: wastewater treatment plants exist almost everywhere and *E. coli* is readily available for synthetic biology.

We propose to design a genetically engineered *E. coli*, Hydrocoli, with an improved natural hydrogen production pathway, using the organic compounds found in wastewaters as substrate. In addition, we planned to implement various features to enable Hydrocoli to perform other tasks related to wastewater treatment, such as signaling metallic contamination, eliminating nitrogen compounds, or hindering hydrogen consumption by methanogenic bacteria. We also plan to set up a programmed death module in order to prevent Hydrocoli proliferation outside the wastewater treatment plant.

This approach may result into a reduction of sludge which otherwise would need to be treated. Besides that, the use of genetically modified organism in wastewater treatment has never been achieved and could potentially increase the efficiency of the process. It could also show people that genetically modified organisms are tools able to improve our environment that should be considered.

The ethical and economical issues of our project and of synthetic biology in general will also be discussed.

In order to tackle this ambitious project in a relatively short time, we have decided to divide our approach in several modules:

- Hydrogen production
- Homologous recombination

- Bacteria programmed death module
- Detection of metal contamination

For each module, we developed to a computerized model in order to analyze the kinetics of reactions prior to the experiments.

Hydrogen production:

The main goal here is to direct the carbon flux through specific pathways, namely the mixed acid fermentation since this pathway leads to hydrogen production. In order to limit the flow through unwanted reactions, we plan to eliminate the enzyme catalyzing key reactions by deleting the corresponding genes. These genes are *ldhA*, *ppc* and *focA*.

E. coli also contains 2 hydrogenases, Hyab and Hybc, which might use the newly produced hydrogen. These two enzymes must obviously be inactivated.

We also plan to over-express several genes that are involved in enhancing the mixed acid fermentation: FNR, which is a global regulator of anaerobic growth, TdcE and PflB, coding for the two pyruvate formate lyase, which catalyze the transformation of pyruvate into formate a key reaction in mixed acid fermentation.

Homologous recombination:

In order to increase the production rate of hydrogen in our Hydrocoli, we plan to delete several genes. We plan to construct these deletions using a method based on the λ phage Red system developed by Datsenko and Wanner (2000).

We plan to develop homologous recombination tools that will be useful to the iGEM community The homologous recombination module focuses on the development of tools that would enable the deletion of any gene on known sequence, using the method mentioned above.

Quorum addiction module:

To avoid escape of Hydrocoli outside the wastewater treatment plant (or wherever they are confined), we plan to implement a system of quorum addiction. The system is composed of a toxin gene, *parE*, and its cognate antitoxin, *parD*. Expression of these genes is under the control of quorum sensing-dependent promoters. In high population density conditions (i.e. in the wastewater treatment plant), Hydrocoli is alive due to the ParD antitoxin production. If Hydrocoli escapes, the population density will drastically drop. These conditions favor the toxin expression and therefore, Hydrocoli will die.

In addition, we thought of possible improvements for Hydrocoli.
Since we considered its use in wastewater treatment, we wanted to equip it with a copper detection system, enabling a change of color of the bacteria when exposed to copper.

In the same perspective, we planned to implement a denitrification module in Hydrocoli. This would be very helpful, since this process is a very important step in wastewaters treatment.
Also, methanogenic bacteria are often found in wastewater treatment plant, and those are able to use hydrogen to form methane, which would obviously decrease the global hydrogen production. To avoid that, we thought of equipping Hydrocoli with adequate molecules to avoid the proliferation of those organism, such as bacteriocins. Bovicin HC5 from *Streptococcus Bovis* seemed an appropriated choice, since it has been shown to substantially decrease methane production in specific context (when mixed with rumenal bacteria).

II. HYDROGEN PRODUCTION

E. coli naturally produces hydrogen through the mixed acid fermentation. Our goal in this part of the project is to increase the H_2 production by modifying the carbon flow through the pathway of the mixed acid fermentation (MAF). The pathways of this fermentation are quite simple. Phosphoenolpyruvate (PEP), which is produced by the glycolysis, is transformed into pyruvate or into oxaloacetate. Those two molecules are then transformed into several products after a chain of reactions. Hydrogen is one of those products. It is produced from formate to regulate the intracellular pH of the bacteria [3].

Here is a simplified global overview of the mixed acid fermentation (fig.1):

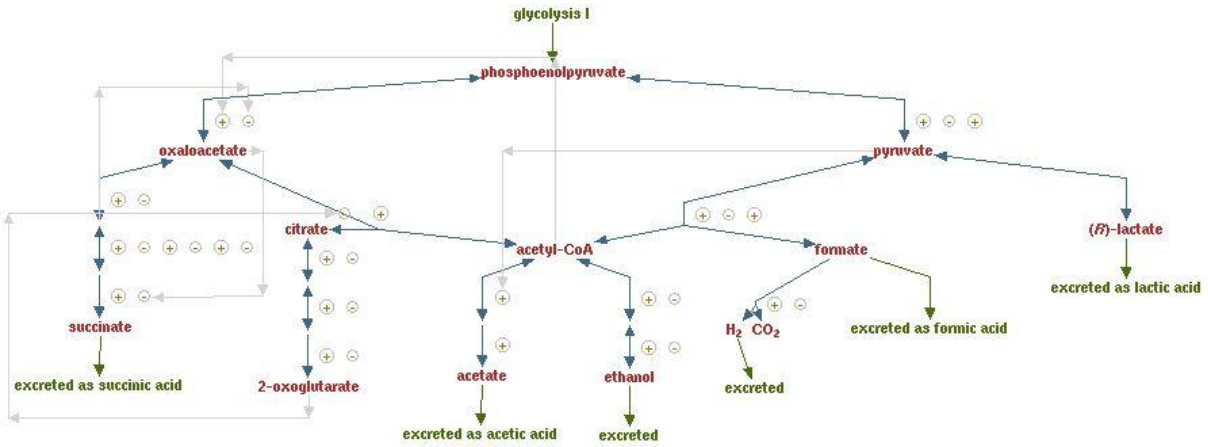


Fig. 1 : Mixed acid fermentation pathway [4].

Each reaction is controlled by one or several enzymes. By overexpressing or deleting specific genes of the MAF pathway, we expect to increase the hydrogen production. The part of MAF pathway in which we are interested is indicated by red lines (fig. 2).

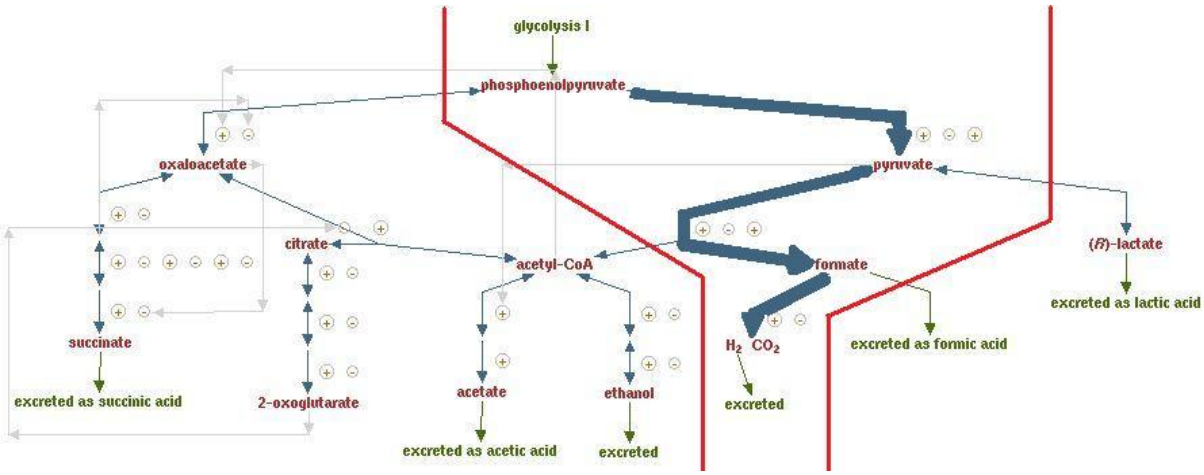


Fig. 2: The interesting pathway for hydrogen production via the mixed acid fermentation

All the reactions crossing the red lines should be avoided or limited, and the three main reactions which lead to hydrogen production should be increased.

The hydrogen is produced from formate by the formate hydrogenase lyase system (FHL), an intracellular membrane-bound complex composed of the formate dehydrogenase (FDH_H) and the hydrogenase 3 [3]. This complex catalyzes the following reaction:

$$\text{Formate} + H^+ \leftrightarrow H_2 + CO_2$$

Hydrogen is not excreted outside of the cell but it is immediately consumed by two uptake hydrogenases, the hydrogenase 1 and 2 [3][4]:

$$\text{A menaquinone} + H_2 + 2H^+ \leftrightarrow \text{A menaquinol} + 2H^+$$

The expression of the FHL system is activated at the level of transcription by the FhlA activator (formate hydrogen-lyase transcriptional activator). Expression of *fhlA* is positively regulated by FNR and repressed by HycA [3]. FNR is the main transcriptional regulator that mediates the transition between aerobic and anaerobic growth through the regulation of hundreds of genes [4]. All the regulations linked to the transformation of formate into hydrogen are shown here (fig.3):

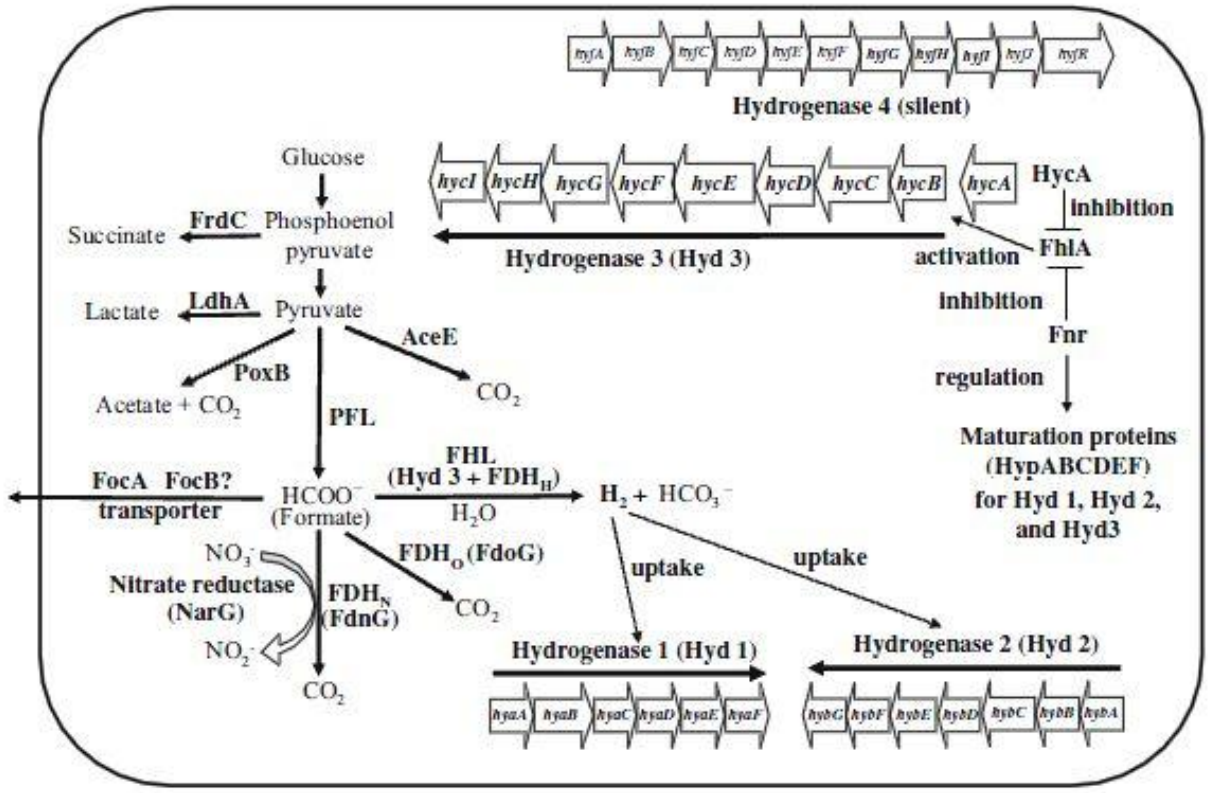


Fig. 3 : Regulation of hydrogen production in *E. coli* [3].

II.1 MAF PATHWAY MODIFICATION: GENES TO DELETE

Several steps of the MAF pathway are irrelevant for hydrogen production (fig. 2). To inactivate these reactions, the genes encoding enzymes that catalyze these reactions have to be deleted using the technique described in the homologous recombination module.

After exhaustive analyzes of the literature, we decided which genes we would overexpress and which we would disrupt.

Here is the list of all the genes that we planned to delete. By using the KEIO Collection database, we checked that none of those genes were essential [6].

1) ppc

The *ppc* gene encodes the phosphoenolpyruvate carboxylase enzyme (Ppc). This enzyme catalyzes the following reaction [4]:

$$\text{Phosphoenolpyruvate} + CO_2 + H_2O \leftrightarrow \text{Oxaloacetate} + \text{Phosphate} + H^+$$

Deletion of the *ppc* gene will therefore prevent the PEP to be transformed into oxaloacetate which is not interesting for our application. As shown on figures 1 and 2, the

left part of the MAF pathway does not lead to hydrogen production. Thus, deletion of *ppc* will inactivate this part of the pathway and all the PEP will be transformed into pyruvate.

2) ldhA

Pyruvate is transformed into (R)-lactate or into formate + acetyl-CoA. The second reaction is interesting for our application. The first reaction is mediated by the D-lactate dehydrogenase (LdhA) which catalyzes the following reaction:

$$NADH + Pyruvate + H^+ \leftrightarrow NAD^+ + (R)\text{-lactate}$$

Deletion of *ldhA* should ensure that all the pyruvate is transformed into formate + acetyl-CoA.

3) focA

The *focA* gene encodes for a formate transporter located in the plasmic membrane. FocA transports formate outside the cell. It has been shown that disabling this transporter increases the intracellular formate concentration [7]. A higher formate intracellular concentration is obviously interesting for hydrogen production.

4) *hyaB* and *hybC*

To prevent hydrogen uptake by the two hydrogenases, HyaB and HybC, we have to inactivate them. Both hydrogenases-encoding genes are located in operons.

The *hya* operon is composed of 6 genes (from *hyaA* to *hyaF*). The *hyaB* gene encodes the largest subunit of the hydrogenase [4]. This subunit is required for the hydrogenase to be functional [4]. Deleting this gene should inactivate the hydrogenase 1 [3].

The second hydrogenase-encoding gene is located in an operon composed of 7 genes (from *hybA* to *hybG*). The *hybC* gene encodes the largest subunit of the second hydrogenase. As for *hyaB*, deleting the gene encoding the largest subunit should inactivate it [3].

1.A MATERIALS AND METHODS

1. Gene deletion by the λ phage Red system

In order to delete the five genes listed above, we used a method based on the λ phage Red system. As this method is described in great details in the 'homologous recombination module', we will briefly summarize it here. This method consists in the production of a gene-specific PCR fragment which contains an antibiotic resistance gene and site-specific recombination sites (FRT) flanking this gene. This PCR fragment is then electroporated in a strain containing the plasmid helper λ (PH λ) (see homologous

recombination module). This plasmid encodes the genes of the Red system. The target gene is replaced by the resistance gene through homologous recombination using the Red system. We worked with two different antibiotic resistances: chloramphenicol (cm) and kanamicine (kan).

The antibiotic resistance cassette is eventually removed by site-specific recombination using the Flp recombinase. This method leads to the 'clean' deletion of the gene of interest without any traces of antibiotic resistance gene. It is also adapted to delete genes in operons since the resistance-FRT cassettes used in this work also contain translation signal (RBS). This allows a proper expression of the genes downstream of the deletion (no polar effect). The primers used deleting the 5 genes of interest are listed in Table 1.

2. Phage P1 transduction

P1vir stock :

Overnight cultures of the donor strain are diluted 100 fold in 20 ml of LB medium supplemented with MgSO4 10^{-2}M and of CaCl2 5.10^{-3}M and grown at 37°C. At an optical density at 600nm of 0.2, 10µl of a P1 vir stock is added (approximately 10^9 pfu/ml). Once the bacteria are lysed, 1/20 (v/v) of chloroform is added and the culture is vigorously shacked. The mix is centrifuged at 4°C at 3,700 g and the supernatant is conserved at 4°C. 1/20 of the total volume of chloroform is added and vigorously shacked, then centrifuged again in the same conditions. The surnageant containing the P1 particles is stored at 4°C.

P1vir transduction :

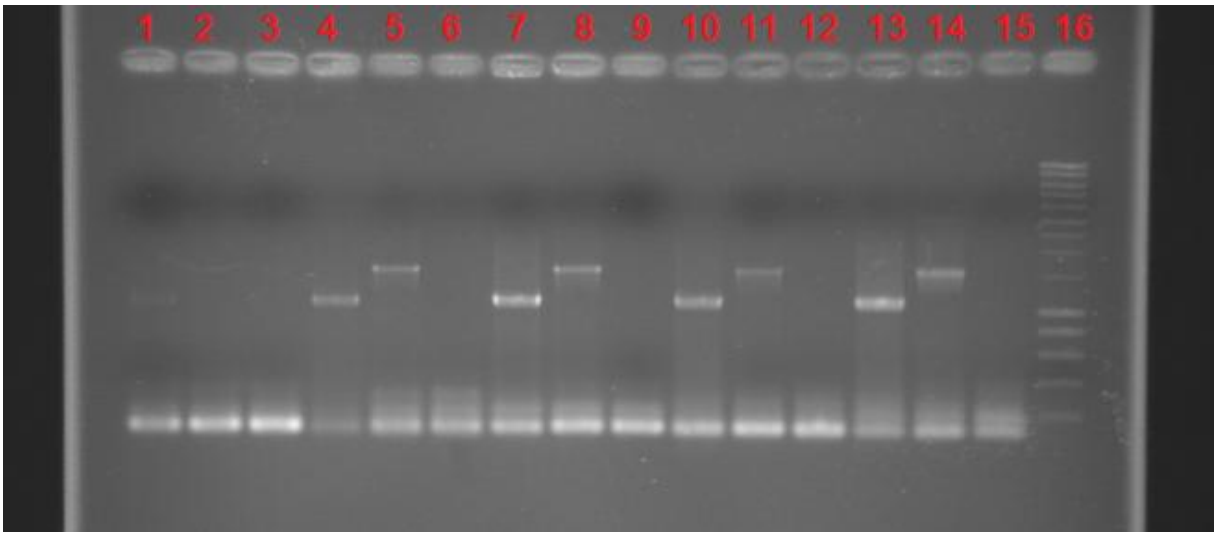
Overnight culture of the recipient strain is pelleted and resuspended in the same volume of MgSO4 10^{-2}M and of CaCl2 5.10^{-3}M. To 100µl of the resuspended pellet, 100µl and 10µl of the P1 lysat is added. The mix is conserved 30 min at 37°C. 3 ml of agar 0.7% is added as well as 100µl of Na citrate 1M. The entire mix is plated on selective plates at 37°C for 16h. The candidates are streaked 3 times on selective plates and a PCR is then performed to confirm the transduction of the marker of interest.

1.B RESULTS

1. Deletion of the *ldhA* gene by homologous recombination

The amplification of the resistance cassettes with the primers specific to our 5 genes of interest by PCR took us a little more than a week. Because of the size of the primers used, we had to try several times the PCR and vary the parameters.

Here is the picture of the electrophoresis of those PCR for the 5 genes:

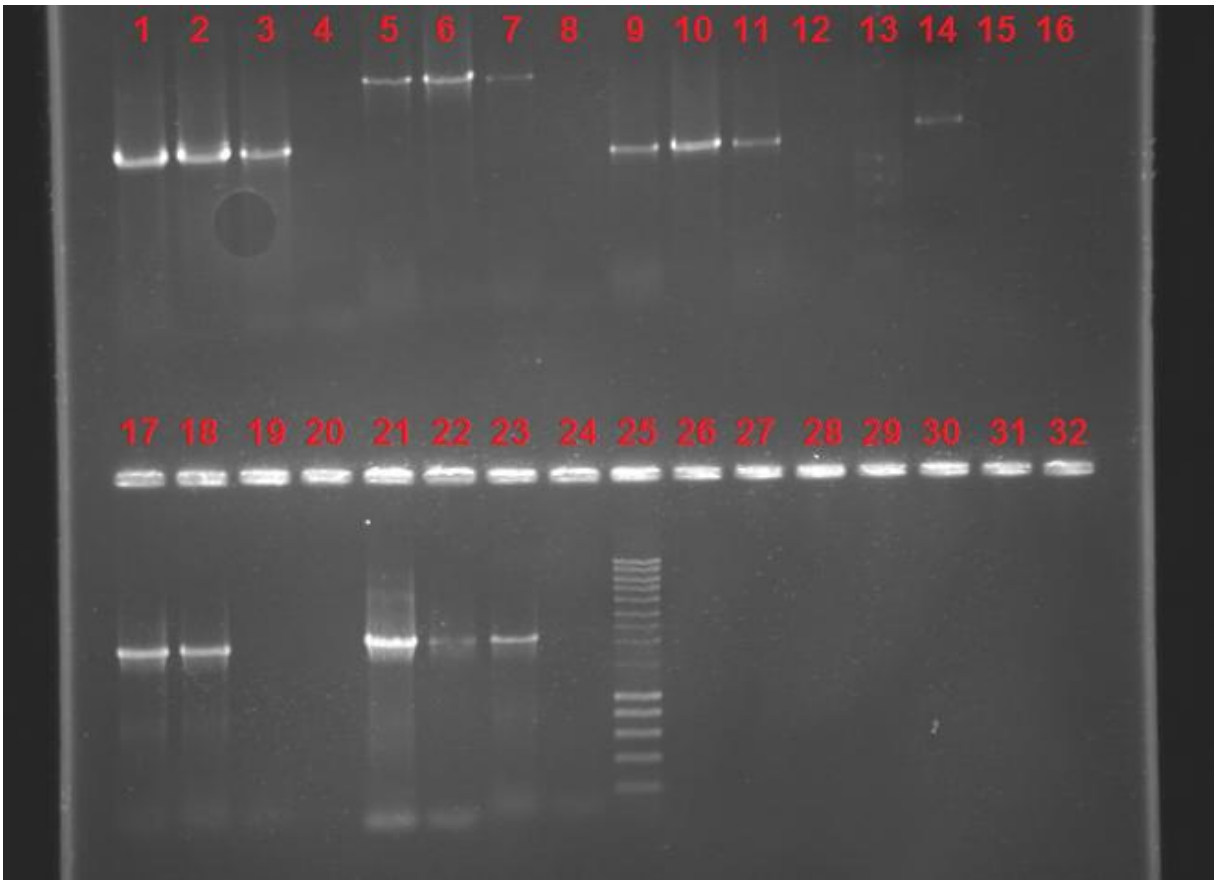


For each genes we tried to obtain both chloramphenicol (about 1100bp) and kanamicine (about 1500bp) resistance cassettes. From left to right we have the genes *ldhA* (1 and 2), *focA* (4 and 5), *ppc* (7 and 8), *hybC* (10 and 11) and *hyaB* (13 and 14). The sinks 3, 6, 9, 12 and 15 are the five negative controls.

Once we obtained these cassettes, it took us about 1 month to obtain our first deletion candidate. The electroporation efficiency of the MG1655/λ strain was not good. We obtained a lot of false-positive deletion candidates (i.e. clones growing on the selective medium). As explained in the homologous recombination module, we had to purify at least three times each candidate (this process takes therefore at least 3 days) before checking the deletion/insertion of the resistance cassette. Using primers complementary to the flanking regions of the genes of interest, PCR were performed on candidate colonies (data shown below). Unfortunately, the candidates were most of the time wild-type for the gene of interest. Our hypothesis is that the resistance cassette was inserted at another location in the genome. The reason for that is unknown since the genes of interest are not essential for *E. coli* viability, at least in the conditions we used.

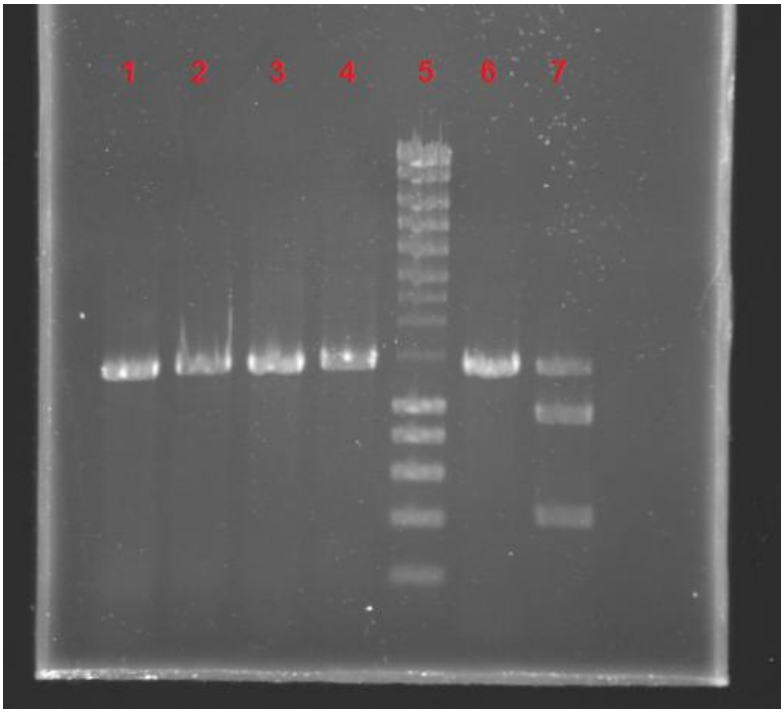
After several weeks of unsuccessful attempts, we finally obtained the MG1655 Δ*ldhA::cm* strain.

Here is the picture of the electrophoresis where we tested different deletion candidates:



The sinks we are interested in are the 4 first ones. The wild type version of the gene *ldhA* is in the third sink. The two first sinks are the deletion candidates for *ldhA* with the cm resistance cassette. The fourth sink is the negative control for this gene. For information, the other sinks are the tests for the 4 other genes: 5 and 6 are the deletion candidates for ppc; 9 and 10 are the deletion candidates for focA; 17 and 18 are the deletion candidates for hybC: 21 and 22 are the deletion candidates for hyaB. Unfortunately, the cm resistance cassette (about 1100 bp) and the gene *ldhA* (990 bp) have approximately the same length. In order to testify if we really had the MG1655 Δ*ldhA::cm* strain, we did a test with a restriction enzyme which cut the gene *ldhA.*

The result of this test is shown here:

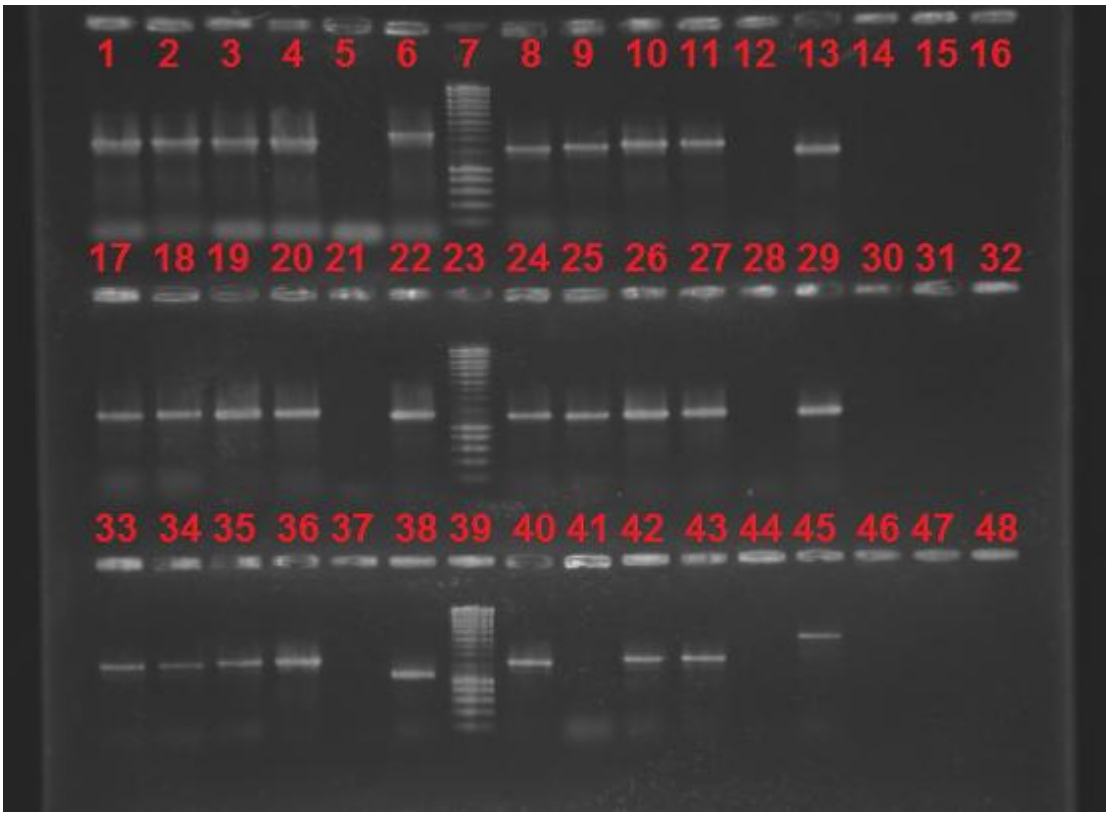


After the purification process, we had 4 candidates for the MG1655 Δ*ldhA::cm* strain. For those 4 candidates, we did a PCR on the gene *ldhA* (not knowing if it really was *ldhA* or the cm resistance cassette that we amplified) and we digest the product of the PCR with an enzyme which cut *ldhA.* The digested gene *ldhA* is in the sink 7, the digested cm resistance cassette is in the sink 6. The four digested candidates are in the 4 first sinks. The result is therefore a success, because the candidates are not digested like *ldhA*. The wild-type version of the gene has been successfully replaced with the cm resistance cassette. We have our MG1655 *ΔldhA::cm* strain.

2. Construction of the MG1655ΔΔ*ppc::kan*, MG1655ΔΔ*focA::kan*, MG1655ΔΔ*hyaB::kan*, MG1655 Δ*ldhA::cm* Δ*ppc::kan*, MG1655 *ΔldhA::cmΔfocA::kan* and MG1655 *ΔldhA::cm*ΔΔ*hyaB::kan* mutant strains by P1 transduction

By that time, we obtained the KEIO strains deleted for our 5 genes of interest. We were thus able to make a P1 stock on these strains (see materials and methods) and to transduce the *Δppc::kan*, *ΔfocA::kan*, *ΔhyaB::kan* and *ΔhybC::kan* markers in the MG1655 wild-type strain and in the MG1655 *ΔldhA::cm* strain. We obtained 6 mutant strains: the single MG1655 *Δppc::kan*, MG1655 *ΔfocA::kan* and MG1655 *ΔhyaB::kan* mutant strains as well as the double MG1655 *ΔldhA::cm Δppc::kan*, MG1655 *ΔldhA::cm ΔfocA::kan* and MG1655 *ΔldhA::cm ΔhyaB::kan* mutant strains. These strains were checked by PCR.

The results are shown here:



For each strain tested, we have: four candidates, one negative control and the wild type version of the gene that is supposed to have been deleted. If the amplified DNA segment of a candidate has the size of the reistance cassette instead of the size of the wild type version of the gene, the candidate is a mutant. Here are the results:

- From 1 to 6 is the test for the MG1655 *ΔhyaB::kan* mutant: the 4 candidates are mutants.
- From 8 to 13 is the test for the MG1655 *ΔldhA::kan* mutant: the candidates in the sinks 10 and 11 are mutants.
- From 17 to 22 is the test for the gene *ldhA* of the double MG1655 *ΔldhA::cm Δppc::kan* mutant: the 4 candidates are mutants.
- From 24 to 29 is the test for the gene *ldhA* of the double MG1655 *ΔldhA::cm ΔfocA::kan* mutant: the 4 candidates are mutants.
- From 33 to 38 is the test for the gene focA of the double MG1655 *ΔldhA::cm ΔfocA::kan* mutant: the candidates in the sinks 35 and 36 are mutants.
- From 40 to 45 is the test for the gene *ppc* of the double MG1655 *ΔldhA::cm Δppc::kan* mutant: the candidates in the sinks 40, 42 and 43 are mutants.

We managed to obtain different double mutant strains, ready for another transduction. Unfortunately, due to the lack of time we were unable to continue our progression and we were also unable to characterize the double mutant strains for growth in different medium, at different temperature,

II.2 MAF PATHWAY MODIFICATION: GENES TO OVEREXPRESS

In order to enhance hydrogen production, we proposed to over-express some genes involved in the hydrogen production. Three steps in the mixed acid fermentation pathway are of interest:

- The transformation of phosphoenolpyruvate into pyruvate:

$$\text{Phosphoenolpyruvate} + \text{ADP} + 2H^+ \leftrightarrow \text{Pyruvate} + \text{ATP}$$

This reaction is present in other pathway. We thus decided not to improve it assuming it will not be the limiting step.

- The transformation of pyruvate into formate:

$$\text{Pyruvate} + \text{Coenzyme A} \leftrightarrow \text{Formate} + \text{Acetyl-CoA}$$

This reaction is catalyzed by two pyruvate formate lyase: PflB and TdcE. The formate is the key molecule for hydrogen production and intracellular elevated formate concentration is needed for an efficient hydrogen production [8].

- The transformation of formate into hydrogen:

$$\text{Formate} + H^+ \leftrightarrow CO_2 + H_2$$

This reaction is under the control of the FHL complex (formate hydrogenase lyase composed of the formate dehydrogenase (FDH_H) and the hydrogenase 3). As stated above, the FHL complex is regulated by FNR, a transcriptional regulator that mediates the transition between aerobic and anaerobic growth, and repressed by HyaC. It has been showed that FHL activity is not the limiting step for hydrogen production. We decided not to over-express the two enzymes of the FHL complex [5].

On the basis of these three reactions, we decided to overexpress the following genes:

1. ***fnr***

fnr is quite an obvious choice for the first gene to overexpress. FNR is the main transcriptional regulator that mediates the transition from aerobic to anaerobic growth [4]. The concentration of the FNR protein is similar under aerobic and anaerobic conditions. The

regulation occurs at the level of FNR activity: in the presence of O_2, FNR is inactivated. Under anaerobic conditions, FNR is functional and activates expression of hundreds of genes involved in anaerobic metabolism. In these conditions, FNR represses genes involved in aerobic metabolism [4]. Over-expressing the *fnr* gene should increase FNR concentration and should thus enhance the anaerobic metabolism. The three reactions of the mixed acid fermentation pathway leading to hydrogen described above should therefore be increased.

2. *pflB*

The *pflB* gene encodes one of the two pyruvate formate lyases which catalyze the transformation of pyruvate into formate [4]. This reaction is the most important one for hydrogen production. Over-expressing *pflB* should increase formate concentration and therefore hydrogen production. Although pyruvate is the substrate for many other reactions, high level of PflB should direct pyruvate to formate synthesis and therefore hydrogen production.

3. *tdcE*

The *tdcE* gene encodes the second pyruvate formate lyase [4]. The reasons to overexpress this gene are the same as for *pflB*.

2.A MATERIALS AND METHODS

To over-express these three genes of interest, we decided to insert them into plasmid pSB1C3 with a strong RBS, a constitutive promoter and a terminator. We decided to work in the BioBrick standard assembly 10 because it is compatible with pSB1C3.

First of all, we had to obtain the three genes to ligate them with the RBS, the promoter and the terminator. Unfortunately, the *fnr* gene contains an EcoR1 restriction site. Therefore, we had to introduce a silent mutation in the sequence of this restriction site and ordered the modified *fnr* on mrgene.com.

The *tdcE* gene was obtained by PCR using specific primers and the wild-type genome of *E. coli*.

The *pflB* sequence contains a restriction enzyme present in the standard assembly 10: Pst1. We could have also introduced a silent mutation in this sequence and ordered it on mrgene.com. However, due to the size of the *pflb* gene (2283 bp) and our limited budget, we were not able to afford it.

The RBS, the promoter and the terminator that were selected are the same for the three genes and are described as being the ‘strongest’ ones received in spring 2010 distribution.

The promoter is: BBa_J23100

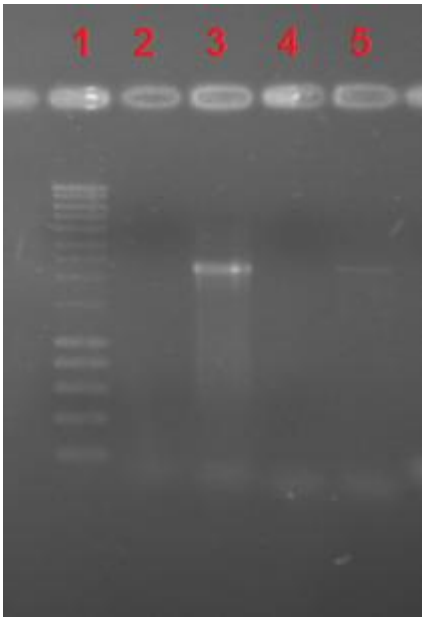
The RBS is: BBa_B0034

The terminator is: BBa_B0024

After obtaining the gene sequences either by PCR or by *in vitro* synthesis, the plan was to ligated them into the plasmid containing the RBS opened with Spe1 and Pst1 in order to clone the genes downstream of the RBS. The genes were also digested by Xba1 and Pst1. The second step was to ligate the part RBS + gene with the promoter. The third step consisted in extracting the construction promoter + RBS + gene and inserting upstream of the terminator. The last step was to extract the promoter+RBS+gene+terminator and insert it into the pSB1C3 plasmid.

Unfortunately, due to lack of time, we were unable to do all these constructs.

We managed to obtain the gene *tdcE* by PCR as we can see here:



The gene *tdcE* is in the sink 3 and 5. The two sinks correspond to two different PCR programs. The sinks 2 and 4 are the negative controls for those PCR.

We digested *tdcE* and inserted it in the pSB1C3 to send it as a BioBrick: BBa_K348000.

2.B GENES OF THE MAF PATHWAY THAT WE DID NOT CONSIDER

Several other genes of the MAF pathway could have been potential candidates for modifications (deletion or overexpression). However, after exhaustive literature readings, we did not consider them.

1. ***hycA***

This gene encodes HycA, a regulator of the hydrogenase 3 in the FHL system [4]. This regulator limits the amount of hydrogenase 3 in the cell. Deleting *hycA* should therefore lead to an increase of the concentration of hydrogenase 3. Note that as stated above, the hydrogenase 3 catalyzes the transformation of formate into hydrogen. We did not consider this candidate since transformation of formate into hydrogen do not appear to be the limiting step for hydrogen production and normal concentration of hydrogenase 3 is fully adequate [5].

2. ***aceE***

This gene encodes a subunit of the pyruvate dehydrogenase complex. This complex catalyzes the following reaction [4]:

$$\text{Pyruvate} + NAD^+ + \text{coenzyme A} \leftrightarrow \text{Acetyl-CoA} + CO_2 + \text{NADH}$$

For hydrogen production, transformation of pyruvate into formate is needed. The AceE enzyme catalyzes a variety of reactions, and deleting the corresponding gene reduces *E. coli* viability [3]. We therefore decided not to delete this gene. We focused on the transformation of pyruvate into formate and to overexpress the *tdcE* gene (see above).

3. ***poxB***

The *poxB* gene encodes a pyruvate oxydase, which catalyzes the transformation of pyruvate into acetate and CO_2 [3]. It has been described that deleting *poxB* does not increase hydrogen production [3].

4. ***focB***

FocB is a formate transporter (the other formate transporter is FocA, see above). Unlike FocA, FocB is less efficient and less specific to formate. We could have deleted *focB* as well but deleting the two formate transporter would have reduced the growth rate [3].

5. ***fdoG* and *fdnG***

fdoG encodes the formate dehydrogenase-O and *fdnG* the formate dehydrogenase-N. Those two formate dehydrogenase catalyze the transformation of formate into $CO_2 + H^+$. The formate dehydrogenase-O is active under aerobic conditions. We can therefore avoid deleting *fdoG* simply because our bacteria will be used under anaerobic conditions. The formate dehydrogenase-N needs a source of nitrate to be active. In the laboratory, it is easy to work on a medium which does not contain nitrogen and we can avoid deleting this gene.

In reality, in a sewage treatment facility, this could be much more problematic. We can propose that our bacteria will be used to produce hydrogen after the nitrogen removal step. Deleting *fdnG* would be necessary if we want to produce hydrogen in a medium that contains nitrogen. Moreover, the formate dehydrogenase-N is a much more effective enzyme than the FHL system. Most of the formate will thus be consumed by the formate dehydrogenase-N instead of being transformed into hydrogen.

Table 1

All the sequences are listed in the directionality 5' -> 3'.

PPC

H1R1: ACCCTCGCGCAAAAGCACGAGGGTTTGCAGAAGAGGAAGAGTGTAGGCTGGAGCTGCTTC

H2R2: ACAGGGCTATCAAACGATAAGATGGGGTGTCTGGGGTAATCATATGAATATCCTCCTTA

LdhA

H1R1: ATCTGAATCAGCTCCCCTGGAATGCAGGGGAGCGGCAAGAGTGTAGGCTGGAGCTGCTTC

H2R2: AGTAGCTTAAATGTGATTCAACATCACTGGAGAAAGTCTTCATATGAATATCCTCCTTA

FocA

For *focA*, we did not try to delete the entire coding sequence because *focA* is located next to *pflB* (one of the genes useful for hydrogen production, see above). The RBS and the promoter of *pflB* are included into the coding sequence of *focA*. We decided to remove only the half of *focA*, leaving the RBS and promoter of *pflB* untouched.

H1R1: GCTGCGGCCAGAATAACTCATCCATACTGCCAGACATACCGTGTAGGCTGGAGCTGCTTC

H2R2: GTTAGTATCTCGTCGCCGACTTAATAAAGAGAGAGTTAGTCATATGAATATCCTCCTTA

HyaB

H1R1: CAGAAACCGAACATCAGCCAGGCAATGAGGATAAACAGGCGTGTAGGCTGGAGCTGCTTC

H2R2: CGTTGTCGCTTTTCTGTTGCATGATGATTCTCCTTCGCTGCATATGAATATCCTCCTTA

HybC

H1R1: GTCAGCAAAATATTGCCGACCCCTAAGACTAAAATACGCAGTGTAGGCTGGAGCTGCTTC

H2R2: TAAAACAAAACGATCATAATCGTCATGAGGCGAGCAAAGCCATATGAATATCCTCCTTA

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Catalyzes the Efflux of Intracellular Potassium but Not ATP - Antimicrobial agents and chemotherapy - June 2008, p. 2247–2249 Vol. 52, No. 6

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III. QUORUM ADDICTION MODULE

III.1 INTRODUCTION

1.A LIST OF USED ABREVIATIONS

abreviation	Signification
HSL	3-oxo-hexanoyl-homoserine lactone
aiiA	HSL degrading protein (BBa_C0060)

In our project, it was important to make sure that Hydrocoli would not be released into the environment of the sewage treatment plant. The QUORUM ADDICTION MODULE ensures that bacteria escaping the sewage treatment plant are killed.

1.B CONCEPTS USED

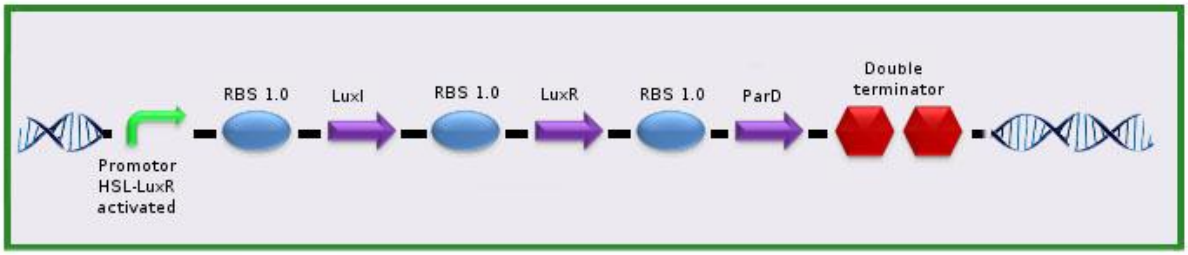
Quorum sensing : The quorum sensing is a mechanism used by bacteria to perceive their own concentration, or the concentration of other species in their environment. To do that, the bacteria secretes homoserine lactones which vary depending on the bacterial species. These molecules control the expression of hundreds of genes such as genes involved in biofilm formation or virulence factor. In the Hydrocoli project, we used two genes belonging to the *lux* operon of *Vibrio fischeri* (*luxR* and *luxI*).

The parDE poison-antidote system : Poison-antidote systems are composed of 2 genes; one encoding a toxic protein and the other encoding its cognate antidote. These systems are abundant in bacterial genomes, although their role in bacterial physiology is still uncertain. When they are located on plasmids, these systems contribute to plasmid stability.

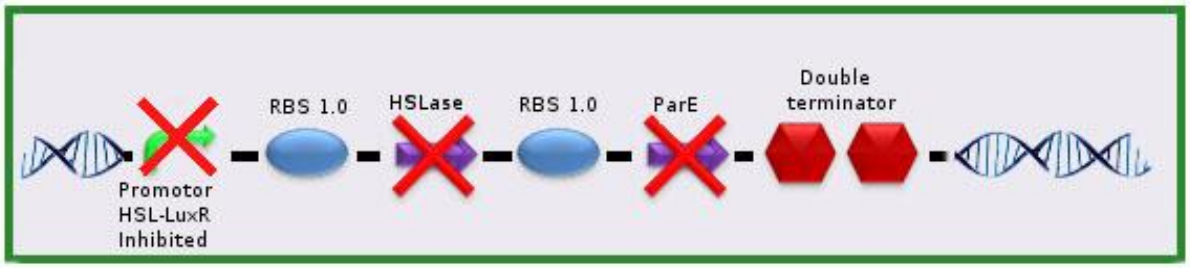
The Quorum addiction module is based on two plasmids that 'interact' with each other. These plasmids, once integrated into the bacterial strain, allow the bacteria to survive in condition in which the bacterial density is high enough. In condition in which the bacterial density decreases, a toxin gene is expressed and the toxin will eventually lead to cell death.

For the construction of this module, BioBricks of cell-cell signaling pathways were used as well as the well-characterized *parDE* poison-antidote system.

The first construction was named the antitoxin plasmid. It contains the 'HSL-LuxR activated' promoter (BBa_R0062), the *luxI* gene (BBa_C0061), the *luxR* gene (BBa_C0062), the *parD* (antitoxin) gene and a double terminator sequence (BBa_B0015).

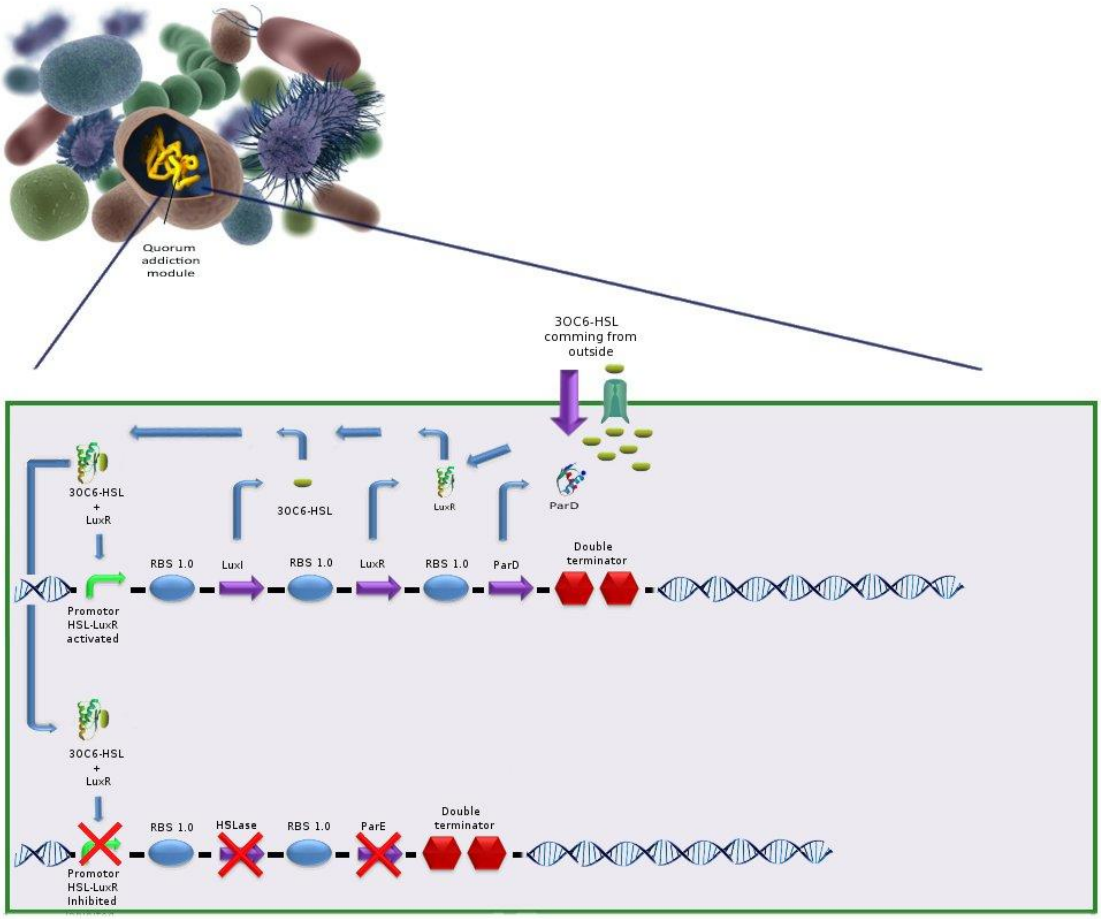


The second construction was named the toxin plasmid. It contains the 'HSL-LuxR repressed' promoter (BBa_R0061), the *aiiA* gene (BBa_C0060), the *parE* (toxin) gene and a double terminator sequence (BBa_B0015).



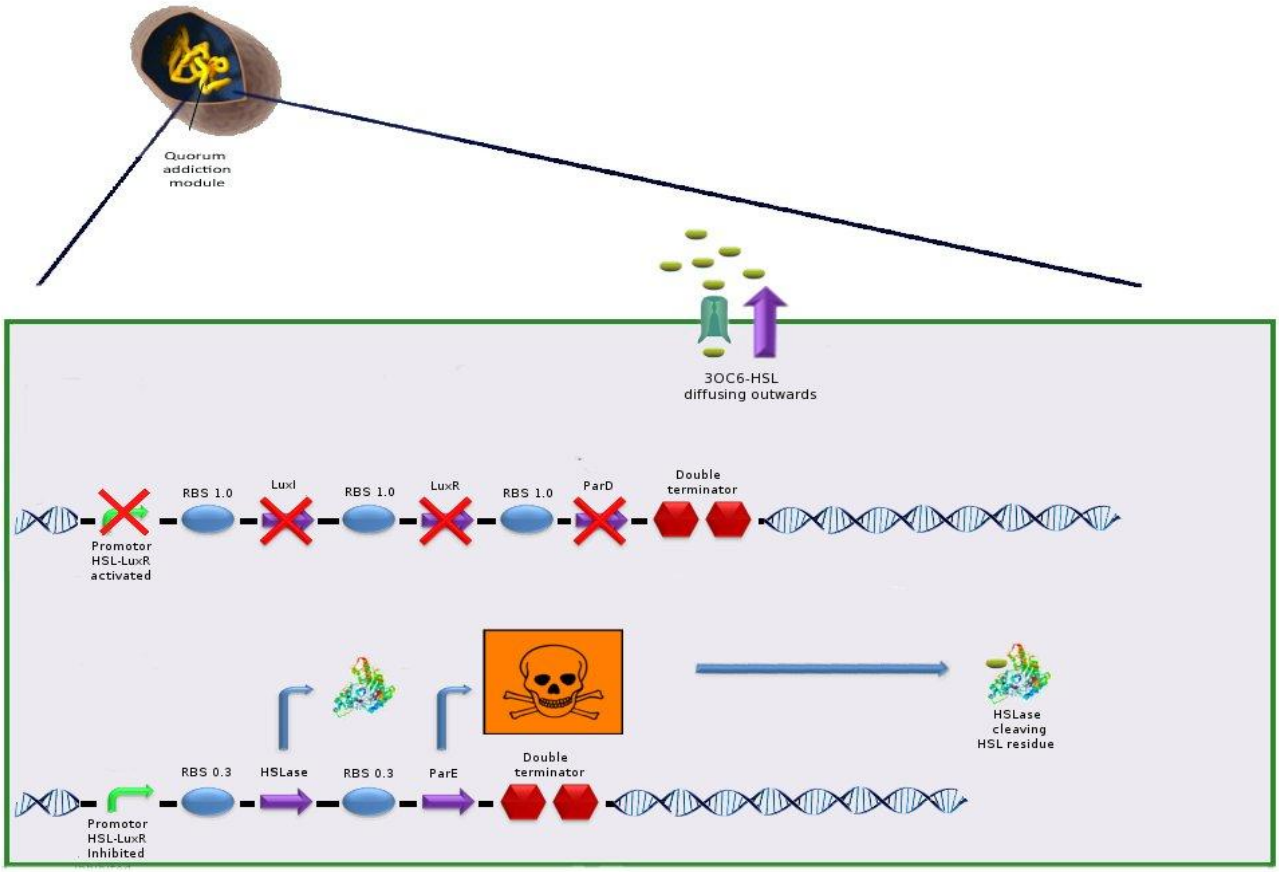
In the sewage treatment plant:

When bacteria are in the sewage treatment plant, the genes of the antitoxin plasmid are expressed to a basal level and the LuxI, LuxR and ParD proteins are produced. Expression of LuxI leads to HSL production. As HSL is able to diffuse 'in and out' of the bacterial cell, both the 'outside' and intracellular HSL concentration will be high. This will have an opposite effect on the antitoxin and toxin plasmids. On the one hand, the LuxR-HSL complex binds to the 'HSL-LuxR activated' promoter and activates the transcription of *luxI*, *luxR* and *parD* genes located on the antitoxin plasmid. This is a positive feedback loop. More and more HSL, LuxR and ParD are produced. On the other hand, the same LuxR-HSL complex has an opposite effect on expression of the genes encoded by the toxin plasmid. It binds to the 'HSL-LuxR repressed' promoter and inhibits the *aiiA* and *parE* gene expression. In these conditions, bacteria are growing 'happily' and do their job in the sewage treatment plan



Out of the sewage treatment plant:

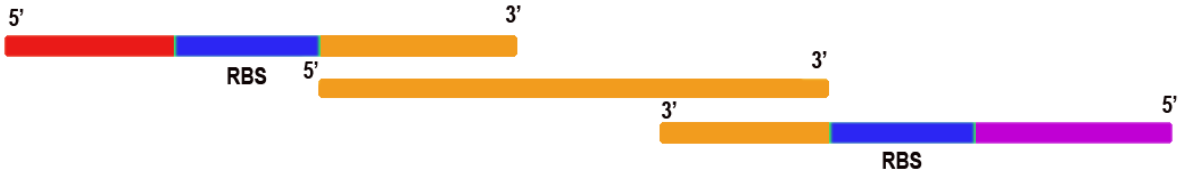
If bacteria escape out of the sewage treatment plant and end up in the outside environment, the HSL concentration will immediately drop. This will lead to a drastic drop of the intracellular HSL concentration and therefore, expression of the antitoxin plasmid genes will be inhibited while that of the toxin plasmid genes will be activated. The ParE toxin will be produced as well as AiiA, an HSL degrading enzyme (*aiiA*, (BBa_C0060)). The ParE toxin will kill the bacteria and the AiiA enzyme will ensure a complete removal of HSL.



III.2 MATERIALS AND METHODS

2.A ASSEMBLY OF GENETIC SEQUENCES

Given the many parts to be assembled, a different technique from the usual technique was used. The assembly of the two constructions was performed by PCR. Primers have been designed to "stick" together two genes (see primer sequences in annex).
The first step was to amplify each part separately.

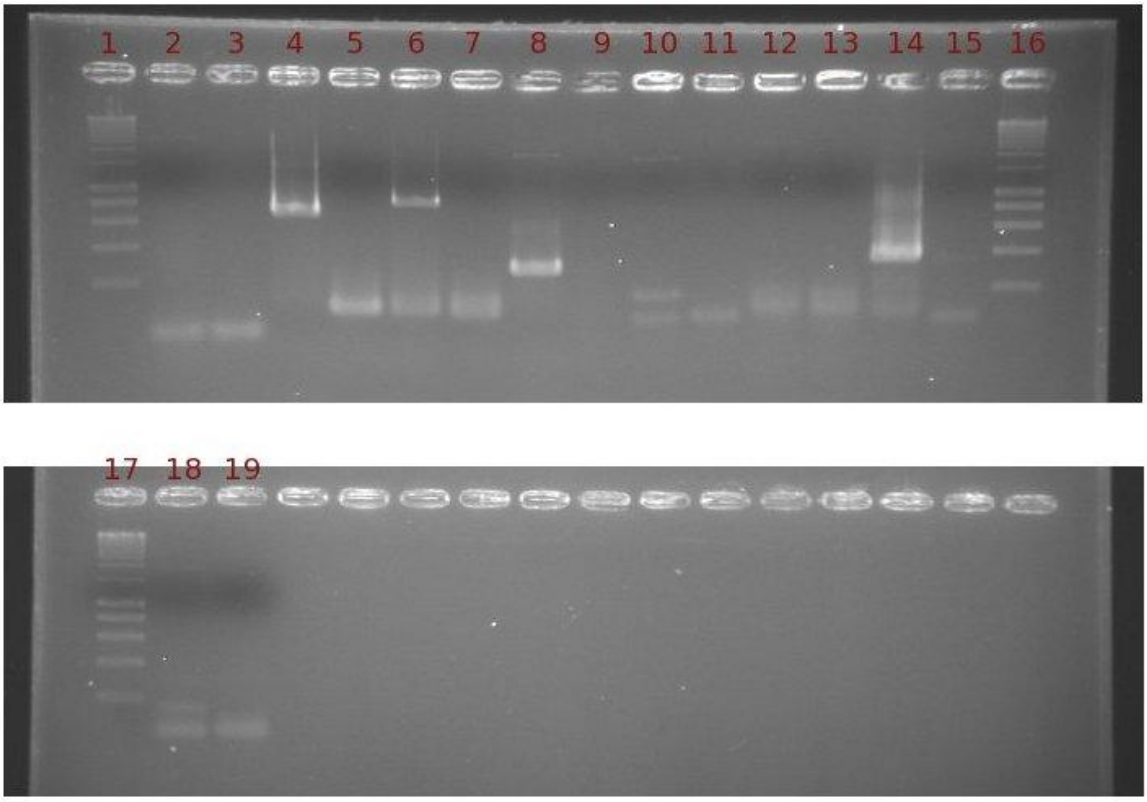


Here (at the top and bottom of the image) the oligos of the first step “stick” to the gene of interest (in orange in the center). After this first PCR we obtain the gene of interest able to “stick” together in the right order. Indeed, each gene has a sticky end with one upstream and another with the downstream.

For the second step we put all genes involved in the construction together and adding just 2 oligos (one with préfix+beginning of the promotor (5’-3’) second with suffix+end of the terminator (3’-5’)) for replicate by PCR all the construction.

III.3 RESULTS

As explained above we started our PCR assembly. The first gel below shows the PCR products (first step) obtained for *luxI*, *luxR*, *parD*, *parE* and the double terminator. The second gel shows the PCR product for *aiiA*.



PCR amplification of the individual parts.
1. SMART ladder ; 2. Lux pR Promoter ; 3. Lux pR Promotor negative control; 4. LuxI ; 5. LuxI negative control ; 6. LuxR ; 7. LuxR negative control ; 8. ParD ; 9. ParD negative control ; 10. Double terminator ; 11. Double terminator negative control ; 12. iiai ; 13. iiai negative control ; 14. ParE ; 15. ParE negative control ; 16. SMART ; 17. SMART ; 18. Double terminator ; 19. Double terminator negative control.

A gel purification was then performed in order to extract the DNA fragment before the second step of PCR assembly.

We were able to obtain PCR amplification

Despite numerous attempts, it was impossible to amplify the promoter by PCR (HSL-LuxR activated (BBa_R0062)). We don't know why it failed, although we tried several annealing temperature and primer concentrations. Because of this problem we couldn't put together the genes of the first construction. As such, it was impossible to proceed with transformation and measurements.

In contrast, for the second construction we succeeded in the assembly of all the genes (see gel below).

However, due to its inherent toxicity, the second construction cannot be transformed in bacteria, the first construction is a prerequisite of the second construction's implementation.

III.4 PRIMER SEQUENCES

4.A ANTIDOTE PLASMID

- Prefix + promoter :

ccccgaattcgcggccgcttctagagacctgtaggatcgtacagg

- Promoter+RBS+LuxI :

gtttgttatagtcgaataaaaaagaggagaaaatgactataatgataaaaaaatcg

cgatttttttatcattatagtcattttctcctctttttttattcgactataacaaac

- LuxI + RBS + LuxR :

cgctttagtagcttaataaaaagaggagaaaatgaaaaacataaatgccg

cggcatttatgtttttcattttctcctcttttttattaagctactaaagcg

- LuxR + RBS + ParD :

catactttaaaaattaataaaaagaggagaaaatgagccgcctgacaatcg

cgattgtcaggcggctcattttctcctcttttttattaattttaaagtatg

- ParD + double terminator :

cagcggggatcgcgcttgaccaggcatcaaataaaacg

cgttttatttgatgcctggtcaagcgcgatccccgctg

- Double terminator + suffix :

ccccctgcagcggccgctactagtatataaacgcagaaaggccc

4.B POISON PLASMID

- Prefix + promoter +RBS + aiiA :

ccccgaattcgcggccgcttctagagttgacacctgtaggatcgtacaggtataataaagaggagaaaatgacagtaaagaag

ctttatttcg

- aiiA + RBS + ParE:

cgctttagtagcttaataaaaagaggagaaattgacggcctacatcctcac

gtgaggatgtaggccgtcaatttctcctcttttttattaagctactaaagcg

-ParE+doubleterminator :

ccgacaggctcaagggctgaccaggcatcaaataaaacg

cgttttatttgatgcctggtcagcccttgagcctgtcgg

- Doubleterminator+suffix :

ccccctgcagcggccgctactagtatataaacgcagaaaggccc

IV. HOMOLOGOUS RECOMBINATION

The aim of this module is to provide the iGEM community with the tools we used to delete genes in *Escherichia coli*. The deletions were performed using the phage λ Red recombinase, as described by Datsenko and Wanner[1]. The toolbox is composed of two plasmid helpers and two resistance cassette BioBricks, adapted to iGEM standards. Basically one could delete a gene with only this toolbox and a couple of primers designed specifically for the target gene.

We think that a simple way to delete genes could be a great asset in the context of designing new biological systems, and thus could be useful to future iGEM participants. Provided that the gene is not essential to the microorganism, the deletion could be done for different purposes, and open up new approaches in iGEM's synthetic biology projects, alongside the BioBricks system. For example, removing a specific gene could enhance a particular metabolic pathway, like we did for the hydrogen production. Moreover, it could be easier to delete a gene hindering the production of a desired product than to overexpress enhancer genes. It could also be useful if a gene is interfering with the function of a BioBrick.

IV.1 HOW IT WORKS

In order to delete a gene, the first step is a PCR using the specific primers and a resistance cassette as template. The fragment is used by the Red recombinase to replace the target gene with the resistance cassette, and the mutants are then selected on an appropriate selection medium. Finally, the resistance gene is removed, so as to allow several deletions on the same strain while avoiding multiple resistances. Before explaining in details those steps, we will first introduce the different elements.

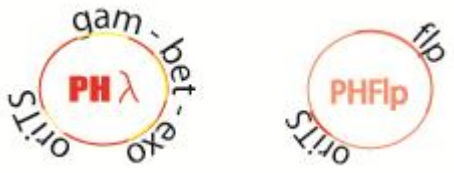
The first plasmid helper (PH λ) contains the three genes of the λ Red recombinase, *γ*, *β*, and *exo*, necessary for homologous recombination. The *γ* gene codes for Gam, which prevents an exonuclease of *E. coli* from inhibiting the homologous recombination, while Exo is a 3'-5' exonuclase preparing the PCR product for the homologous recombination. The product of *β*, Bet, is the recombination enzyme.

The second plasmid helper (PHFlp) contains the *flp* gene coding for the Flippase recombination enzyme (Flp). The enzyme recognizes small sequences called Flippase recognition target (frt) , and, in a way similar to the Cre-Lox systems, causes a recombination between the frt sites. In this case it is used to remove a frt-flanked sequence from genomic DNA.

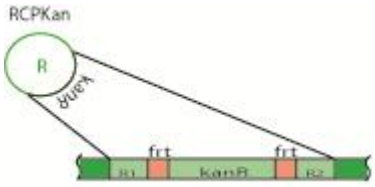
Both plasmid helpers are low copy thermosensitive replicons, which means the plasmids are not replicated during cell division above a certain temperature. Thus the plasmids are easily

[1] *One-step inactivation of chromosomal gene in Escherichia coli K-12 using PCR products*, K.A. Datsenko and B.L. Wanner (2000)

cured after the gene deletion. The expression of the different genes is regulated by thermal induction at 42°C.



The resistance cassettes plasmids (RCP) are plasmids with a resistance gene flanked by frt sequences. There are two resistance cassette plasmids, one with a kanamycin resistance marker (RCPKan) and the other with a chloramphenicol resistance marker (RCPCm)

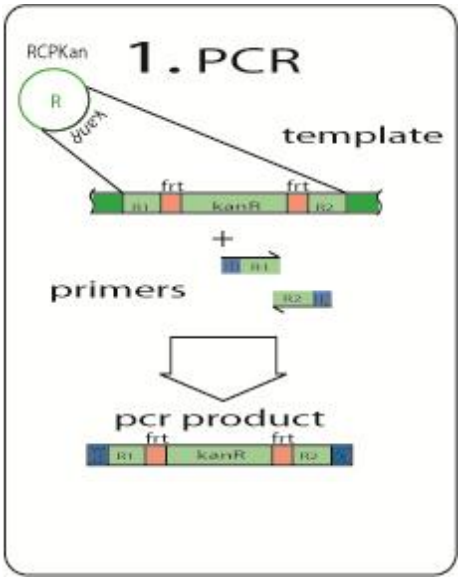


The primers contain a constant part, and a variable part according to the target gene. The constant part is the priming sequence for the resistance gene PCR amplification, and the variable part is homologous to the sequence bordering the target gene.

With these elements, you can achieve a gene deletion in three main steps.

1.A STEP 1: PCR

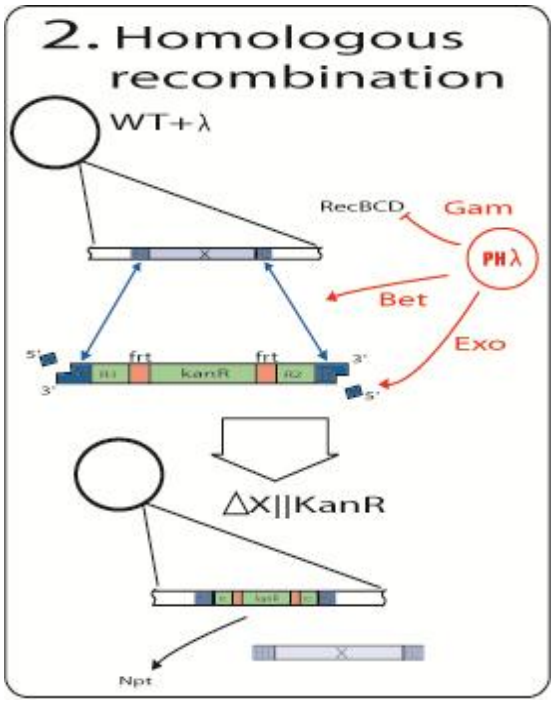
The first step consists of a PCR to produce the sequence which will replace the target gene . By using the frt-flanked resistance gene carried by the RCP as template, and the primers designed especially for the target gene, the product is a sequence which can be recombined



with the Red recombinase.

1.B STEP 2: HOMOLOGOUS RECOMBINATION

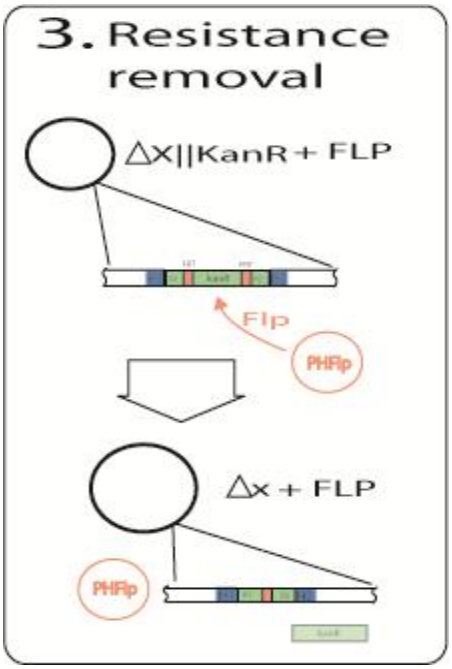
A strain containing the PH λ is then electroporated with the purified PCR products. After



heat induction, Gam, Bet and Exo execute the homologous recombination.

1.C STEP 3: RESISTANCE ELIMINATION

After selection on an appropriate selection medium, the mutant strain is transformed with the PHFlp using the TSS[2] method. Finally, the resistance marker is eliminated and the PHFlp is cured by growth at 42°C.

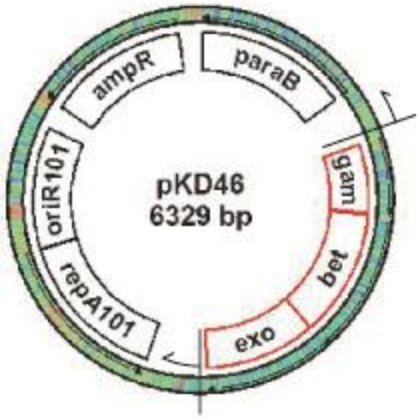


IV.2 PARTS DESIGN

PH λ and PHFlp are derived from the Red recombinase plasmid pKD46[3]. We wanted to adapt them to at least one iGEM standard, and to change the arabinose inducible promoter with a thermo-inducible promoter.

[2] Transformation and Storage Solution

[3] See http://www.ncbi.nlm.nih.gov/nuccore/15554345?report=genbank for features and complete sequence.



For PH λ we chose the RCF 21, as it needed the fewest changes.PKD46 was amplified using primers bordering the Red operon (γ, β, and exo genes), extended with RCF21 prefix and suffix. The idea was to preserve the thermosensitive replication origin (repA101 and oriR101) and the ampicillin resistance while removing the Red operon. We amplified by PCR gam and bet, with RCF 21 prefixes and suffixes, and we ordered exo synthesis[4] with a silent mutation at the *Eco*R1 restriction site, to make it compatible with RCF 21 and 10. The Red operon is recreated with proper thermoinducible promoter (BBa_K098995), RBS (B0034) and terminator (B0024) using the BioBrick system.

The primer sequences are given below, with RCF21 prefix and suffix in green.

pKD46 :

pKD46_RCF21_for : cccGGATCCTAACTCGAGCGCATCCTCACGATAATATC

pKD46_RCF21_rev : cccAGATCTCATGAATTCTATGGCATAGCAAAGTGTGA

gam :

gam_RCF21_for : cccGAATTCATGAGATCTATGGATATTAATACTGAAAC

gam_RCF21_rev : cccCTCGAGTTAGGATCCTTATACCTCTGAATCAATAT

[4] Synthetized by Mr Gene, http://mrgene.com/desktopdefault.aspx/tabid-2/

bet :

bet_RCF21_for : cccGAATTCATGAGATCTATGAGTACTGCACTCGCAAC

bet_RCF21_rev : cccCTCGAGTTAGGATCCTCATGCTGCCACCTTCTGCT

Three C's were added to the end of prefixes and suffixes to facilitate digestion by restriction enzymes (endonucleic enzymes)

We also started from pKD46 for PHFlp, except that we chose RCF 10. The primers used for the pKD46 PCR were the same except for the RCF 10 prefix and suffix extensions. We also conducted a PCR site-directed mutagenesis on the pKD46 PCR fragment to eliminate a *Spe*I restriction site in the oriR101 sequence. The flp gene was synthesized to be compatible with RCF10 and 21, then put together with the pKD46 fragment, promoter, rbs and terminator.

The primer sequences are given below, with RCF10 prefix and suffix in green.

pKD46_RCF10_for : cccTACTAGTAGCGGCCGCTGCAGCGCATCCTCACGATAATATC

pKD46_RCF10_rev : cccCTCTAGAAGCGGCCGCGAATTCTATGGCATAGCAAAGTGTGA

For the RCPs, we used primers with R1 and R2 priming sites, extended with RCF10 prefix and suffix, to amplifiy the kanamycin and chloramphenicol resistance cassettes. The same primers are used for both resistance cassettes, as the R1 and R2 sequences are the same. The templates used are pKD4 for kanamycin resistance cassette and pKD3 for chloramphenicol resistance cassette.

The PCR fragments are then inserted in pSB1C3 plasmid backbones.

The primer sequences are given below, with RCF10 prefix and suffix in green.

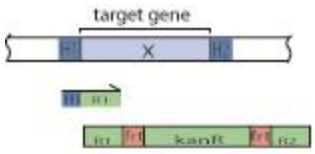
RCF10prefix_R1 : cccGAATTCGCGGCCGCTTCTAGAGGTGTAGGCTGGAGCTGCTTC

RCF10suffix_R2 : cccCTGCAGCGGCCGCTACTAGTACATATGAATATCCTCCTTA

IV.3 PROTOCOLS

3.A RESISTANCE GENE PCR:

The primers must be designed by adding a ~40pb homologous sequence (H) to a resistance gene priming site (R). The homologous sequence must border the sequence to be deleted. The forward primer will be H1R1 and the reverse primer H2R2.



R1 and R2 sequences are given below:

R1 (forward): GTGTAGGCTGGAGCTGCTTC

R2 (reverse): CATATGAATATCCTCCTTA

For instance, here is the sequence of the *ldhA* gene (in blue):

TAGCTGTTCTGGCGTAACAGCAATTTTGTCATTACTTACACATCCCGCCATCAGCAGGCTTAGCGCAA
CAAACGCGGCTACTTTCTTCATTGTGGTTCTCAATTACAGTTTCTGACTCAGGACTATTTTAAGAATAG
AGGATGAAAGGTCATTGGGGATTATCTGAATCAGCTCCCCTGGAATGCAGGGGAGCGGCAAGATTA
AACCAGTTCGTTCGGGCAGGTTTCGCCTTTTTCCAGATTGCTTAAGTTTTGCAGCGTAGTCTGAGAAA
TACTGGTCAGAGCTTCTGCTGTCAGGAATGCCTGGTGCCCGGTAAACAGCACGTTGTGGCAGGCAG
ACAGGCGACGGAATACGTCATCCTGGATCACGTCGTTGGATTTATCTTCAAAGAATAGATCGCGTTC
GTTCTCATACACGTCCATACCCAACGAACCAATTTTCTGATTTTTCAGCGCTTCAATTGCTGCCTGAGA
ATCAATCAATGCACCGCGACTGGTATTGACGATCATCACGCCATTTTTCATCTGTTCGAAGGCGGCTT
CGTTCAACAGATGATAGTTTTCCGGTGTCAGCGGGCAGTGCAGAGAGATAACGTCTGATTCAGAGA
ACAGGGTTGGCAGATCGACATACTCCACACCGAGTTCCAGCGCCGCTGCACTTGGATACGGATCGAA
CGCCAGCAGACGCATACCAAAACCTTTCAGAATGCGCAGCATCGCCACACCGATTTTACCGGTACCG
ATAACGCCTGCCGTTTTGCCATACATAGTAAAGCCGGTCAGACCTTCCAGAGAGAAGTTAGCATCAC
GGGTACGCTGATACGCGCGGTGAATACGGCGGTTCAGCGTCATCATCATACCGATGGCGTGTTCAGC
AACGGCCTCTGGATCATAGGCTGGAACACGGACTACTTTCAGCCCCAGTTCTTTTGCCGCGTCAAGGT
CGACGTTATTGAAACCGGCACAGCGCAGGGCGATATATTTAACGCCGTGCTTTTTCAGCTCTTCCAGC
ACCGGGCGGCTGCCGTCATCGTTTACGAAAATACATACCGCTTCGCAGCCATTGGCAGTTTTAGCGG

TTTTTTCCGTCAGCAGAAAGTCAAAAAATTCCAGCTCAAAGCCAAAGGACTCGTTCACCTGTTGCAGG
TACTTCTTGTCGTACTGTTTTGTGCTATAAACGGCGAGTTTCATAAGACTTTCTCCAGTGATGTTGAAT
CACATTTAAGCTACTAAAAATATTTTACAAAATTTCAAATTTAATTGAAAGCTATGGCGATATTGAAA
AATTCATCAACAACTATGCTTAGTGTAGGCGCAACCTTCAACTGAACGGTTAAACATGCCACAATACC
CGTATTGAATGCTTAATTTTTCGCTAAATCAGGATATTA

If we take 40pb homologous sequence extension (in green), the primers associated to ldha are the following :

H1R1 (forward) :

ATCTGAATCAGCTCCCCTGGAATGCAGGGGAGCGGCAAGAGTGTAGGCTGGAGCTGCTTC

H2R2 (reverse) :

AGTAGCTTAAATGTGATTCAACATCACTGGAGAAAGTCTTCATATGAATATCCTCCTTA

PCR were achieved with Promega® PCR kit (catalog #M8305) with 5% DMSO using the following thermocycler program :

Deletion : PCR program

94°C 5 min.

94°C 1 min.
55°C 30 sec.
72°C 2 min.
} 5 x

72°C 10 min.
94°C 5 min.

94°C 1 min.
62°C 30 sec.
72°C 2 min.
} 30 x

72°C 10 min.

PCR products are then gel-purified. To complettely eliminate the DNA template, digestion with DpnI and a second gel-purification are performed on the PCR product.

3.B HOMOLOGOUS RECOMBINATION

First, MG1655 transformants with PHλ are grown in LB with ampicillin at 30°C (40ml/deletion) to an OD_{600} ~ 0,6. The culture is then incubated with agitation at 42°C for 20-30min. (depending of the volume of culture). For volume greater than 120ml, the culture should be split into smaller volumes to ensure a homogenous heating to 42°C.

Next the transformants are made electrocompentent by cooling on ice for 20-30 min , concentrating 100-fold and washing 4 times with ice-cold water.

50 µl of cells are electroporated with 10-100 ng of PCR product, then 1 ml of LB is added to the shocked cells. The cells are incubated 1 h at 37°C, then spread on agar plates with kanamycin or chloramphenicol to select recombinants.

After first selection, colonies were isolated by streaking on agar medium with the appropriate antibiotic, and incubated 24 h at 37°C. This is done three times, to avoid undisrupted surnumerous chromosome copies, and after the third isolation the recombinants are PCR tested for the loss of the targeted gene.

3.C RESISTANCE GENE REMOVAL

Recombinants are grown in 5ml LB + kanamycin/chloramphenicol at 37°C to DO_{600}~0,3, then 1 ml /deletion are centrifuged (3250 x g, 7 min). The pellet is resuspended in 100 µl /deletion of 1 X TSS[5].

Mix 100 µl of resuspended pellet with ~100µg of PHFlp, incubate 30 min on ice, then add 100 µl LB + kanamycin/chloramphenicol. Incubate 1 h at 30°C, then spread 50 µl on agar plate with ampicillin and incubate overnight at 30°C.

Inoculate in LB + ampicillin + kanamycin/chloramphenicol and incubate overnight at 30°C. Spread on agar plate without antibiotic and incubate one night at 42°C.

Streak successively on agar plate with ampicillin, on agar plate with kanamycin/chloramphenicol, and on agar plate without antibiotics. PCR-test colonies grown on the agar plate without antibiotic to check for resistance removal. If the resistance has been correctly removed and the PHFlp cured, colonies should grow only on the antibiotic-free medium.

3.D PCR VERIFICATIONS

The verifications for correct recombination and resistance gene removal are achieved

[5] 1 X TSS = 10% PEG (wt/vol) , 5% (DMSO)vol/vol, $MgCl_2$ 50 mM, LB

through PCR, using gene-specific primers. The primers were designed with priming sites 180-200 pb away from the borders of the targeted gene, so that in case of a successful resistance gene removal, the amplified PCR fragments would be long enough to be easily spotted after electrophoresis.

IV.4 RESULTS AND DISCUSSION

While we succeeded in deleting genes for the H_2 production part, with a method very similar to the one exposed above, this part of the project remained mostly theoritical. On the workbench, we first focused on the other parts of the project, our priority being gene deletions with the tools available.

In addition, we encountered many difficulties in putting together the different elements in the plasmid backbones. The lack of time prevented us from achieving any usable parts for the toolbox we proposed. Even though, we thought it interesting enough to present what we initially wanted to do.

V. MODELISATION

V.1 OF H_2 MODULE

1.A QUESTIONS FROM THE WETLAB PART

Questions asked by the dihydrogen module team were general: how much would the deletion of the whole, or part, of the proposed genes increase the amount of dihydrogen produced?

To answer these questions the metabolic pathway of mixed acid fermentation had to be modeled before we could, after elimination of equations for part of the pathway, observe the increase in term of dihydrogen production.

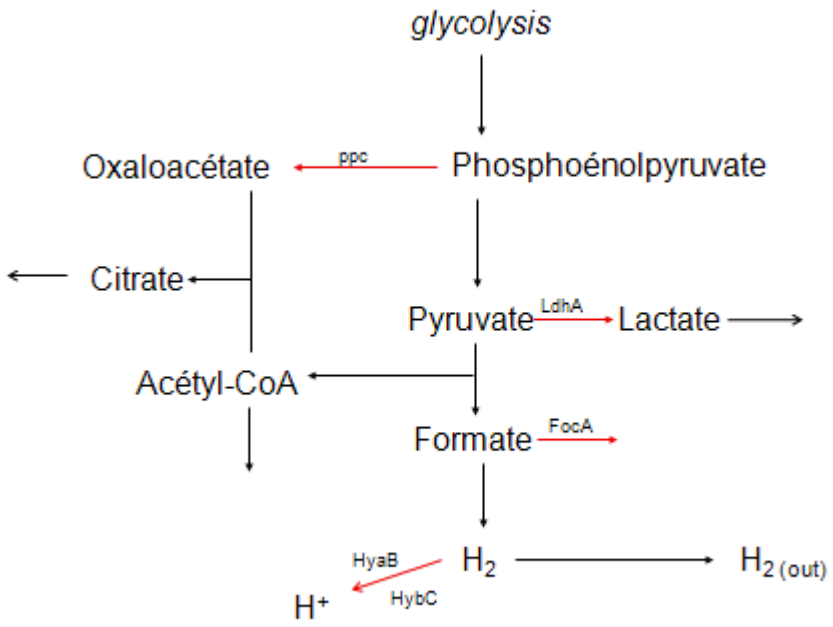


Figure 1. In red, reactions catalyzed by enzymes whose deactivation should increase H_2 production; the gene name corresponding to the enzyme is put on top of the arrow. We didn't include all the genetic regulations in that figure, as they are not relevant to our modeling effort.

1.B INITIAL EQUATIONS AND FURTHER ENHANCEMENTS

We posited the differential equations expressing the variation in the main intermediates of the metabolic pathway vs time. Generally speaking, these equations comprise a creation

term (positive), representing the formation of this intermediate from a substrate, and from one or several destruction terms (negative) that stand for the transformation of this intermediate in the next product.

We distinguish two types of creation or destruction terms.
Linear terms are used when a reaction is assumed spontaneous and non-catalyzed. They are of the *coefficient.reagent concentration* type.
Then the big category of non-linear terms, used in more complex situations where enzymes catalyze the reaction. In the H_2 module, these non-linear terms are Michaëlis-Menten like, which is the typical kinetic of enzyme-catalyzed reactions.
In the next section, we will talk about other non-linear terms, used when we model systems with important genetic regulations.

Here are, the first equations which represent the metabolic pathway of the mixed acid fermentation.

$$\frac{d(PEP)}{dt} = v_0 - v_1 \frac{PEP}{PEP + K1} - v_2 \frac{PEP}{PEP + K2}$$

v0 is a constant of production of the phosphoenolpyruvate by glycolysis.

The two next terms are enzymatic terms of first order, characterizing the catalyzed reaction ADP + PEP ⇔ ATP + PYR + 2H$^+$ (v1) andet la réaction catalysée PEP + CO_2 + H_2O ⇔ OXA + P + H$^+$ (v2)

$$\frac{d(PYR)}{dt} = v_1 \frac{PEP}{PEP + K1} - v_3 \frac{PYR}{PYR + K3} - v_4 \frac{PYR}{PYR + K4}$$

The first term is the production of pyruvate, from phosphoenolpyruvate

The second term (v3) is a term of Michaëlis-Menten for the catalyzed reaction PYR+ CoA ⇔ FOR + AcCO.

The third term (v4) is once again a non-linear term representing the catalyzation of pyruvate in lactate, following the reaction NADH + PYR + H$^+$ ⇔ LAC + NAD$^+$

$$\frac{d(FOR)}{dt} = v_3 \frac{PYR}{PYR + K3} - v_5 \frac{FOR}{FOR + K5} - v_6 \frac{FOR}{FOR + K6}$$

The first term is the formation of the formate, from pyruvate.

The second term (v5) represents the catalysation of $FOR + H^+ \Leftrightarrow CO_2 + H_2$.

The third term (v6) represents facilitated diffusion of formate, by specific and non-specific permeases. It's again an enzymatic reaction, with a typical non-linear kinetic.

$$\frac{d(AcCO)}{dt} = v_3 \frac{PYR}{PYR + K3} - v_7 \frac{AcCO.OXA}{OXA.AcCO + K7}$$

The first term is the formation of acetyl-CoA from pyruvate.

The second (v7) is a non-linear term of the second order in OXA and AcCO, representing the reaction $OXA + AcCO + H_2O \Leftrightarrow CIT + CoA + H^+$

$$\frac{d(OXA)}{dt} = v_2 \frac{PEP}{PEP + K3} - v_7 \frac{AcCO.OXA}{AcCO.OXA + K7}$$

The first term is the apparition of oxaloacetate from PEP

The second term is a non-linear term of the second order for OXA and AcCO, representing the reaction $OXA + AcCO + H_2O \Leftrightarrow CIT + CoA + H^+$

$$\frac{d(CIT)}{dt} = v_7 \frac{AcCO.OXA}{OXA.AcCO + K7} - v_8 \frac{CIT}{CIT + K8}$$

The first term is the production of citrate from acetyl-CoA and oxaloacétate

The second term is the consumption of citrate, in the following reaction.

$$\frac{d(LAC)}{dt} = v_4 \frac{PYR}{PYR + K4} - v_9 \frac{LAC}{LAC + K9}$$

The first term is the production of lactate from pyruvate.

The second term (v9) is the facilitated diffusion term for lactate.

$$\frac{d(H_2)}{dt} = v_5 \frac{FOR}{FOR + K5} - v_{10} \frac{H2}{H2 + K10}$$

The first term is the production of H_2 from formate.

The second term is the of H_2, for example by hydrogenases

We then realized the importance of an equilibrium term in the last reaction leading to the formation of hydrogen (FOR + H^+ ⇔ CO_2 + H_2.),this product can accumulate, which encourages the reverse reaction.
We also refined the kinetic of the reaction OXA + AcCO+ H_2O ⇔ CIT + CoA + H^+, using a non-linear term which considers the concentration and Michaëlis-Menten constant specific of each substrate.
Finally, and to more easily observe the benefit of each deletion, we added an equation modeling the output speed of H_2 diffusing out of the bacteria.
Here are the final equations :

$$\frac{d(PEP)}{dt} = v_0 - v_1 \frac{PEP}{PEP + K1} - v_2 \frac{PEP}{PEP + K2}$$
$$\frac{d(PYR)}{dt} = v_1 \frac{PEP}{PEP + K1} - v_3 \frac{PYR}{PYR + K3} - v_4 \frac{PYR}{PYR + K4}$$
$$\frac{d(FOR)}{dt} = v_3 \frac{PYR}{PYR + K3} - v_5 \frac{FOR}{FOR + K5} + v_{5r} \frac{H_2}{H_2 + K5r} - v_6 \frac{FOR}{FOR + K6}$$

Where the term in v5r is the equilibrium term characterizing the reverse reaction of the H_2 production. This term has been added to account for the balance of the reaction, and avoid an irrealist accumulation of H_2 in the cell.

$$\frac{d(AcCO)}{dt} = v_3 \frac{PYR}{PYR + K3} - v_7 \frac{AcCO.OXA}{K7 + K7a.AcCO + K7b.OXA + AcCO.OXA} - v_{11} \frac{AcCO}{AcCO + K11}$$

Where the term (v7) is a modification of the preceding term in v7, modeling a more general kinetic, to better take into account the differences between the two substrates.

Where the second term (K7b) is a term of utilization of acetyl-CoA in later reactions.

$$\frac{d(OXA)}{dt} = v_2 \frac{PEP}{PEP + K2} - v_7 \frac{AcCO.OXA}{K7 + K7a.AcCO + K7b.OXA + AcCO.OXA}$$

With the same modification than above.

$$\frac{d(CIT)}{dt} = v_7 \frac{AcCO.OXA}{K7 + K7a.AcCO + K7b.OXA + AcCO.OXA} - v_8 \frac{CIT}{CIT + K8}$$

With the same modification than above.

$$\frac{d(H_2)}{dt} = v_5 \frac{FOR}{FOR + K5} - v_{5r} \frac{H_2}{H_2 + K5r} - v_{10} \frac{H_2}{H_2 + K10} - v_{out}.H2$$

$$\frac{d(H_{2out})}{dt} = v_{out}.H2$$

RESULTS

Such a differential equation system can't be analytically solved. We had to use a specialized software able to numerically integrate the system. To do that, we used the XPP software, specifically designed to model biological systems. This software allows us to study the evolution of each variable depending on time. It also has an extension, Auto, able to draw stability diagrams of the systems.

At first, we only vary the parameters, depending on the expected wetlab results. For example, v2=0 means the PPC gene has been successfully Knocked Out (see table 1 for the corresponding between genes deletions and the parameters.). We can see, in the diagram 1, the theoretical effects of such a deletion on the intra-cellular concentration of dihydrogen at the steady-state: it's almost doubled (from 0.057 to 0.118). In the same way, we put each parameters to a nul value, alternately and in a combined way, to observe the effects of the deletions and their optimal combination.

Genes to be desactivated	Corresponding parameters
Ppc	v2
LdhA	v4
FocA	v6
HyaB et HybC	v10

Table 1. Correspondance between experimentally Knock-Out genes and the parameters to be set at null value.

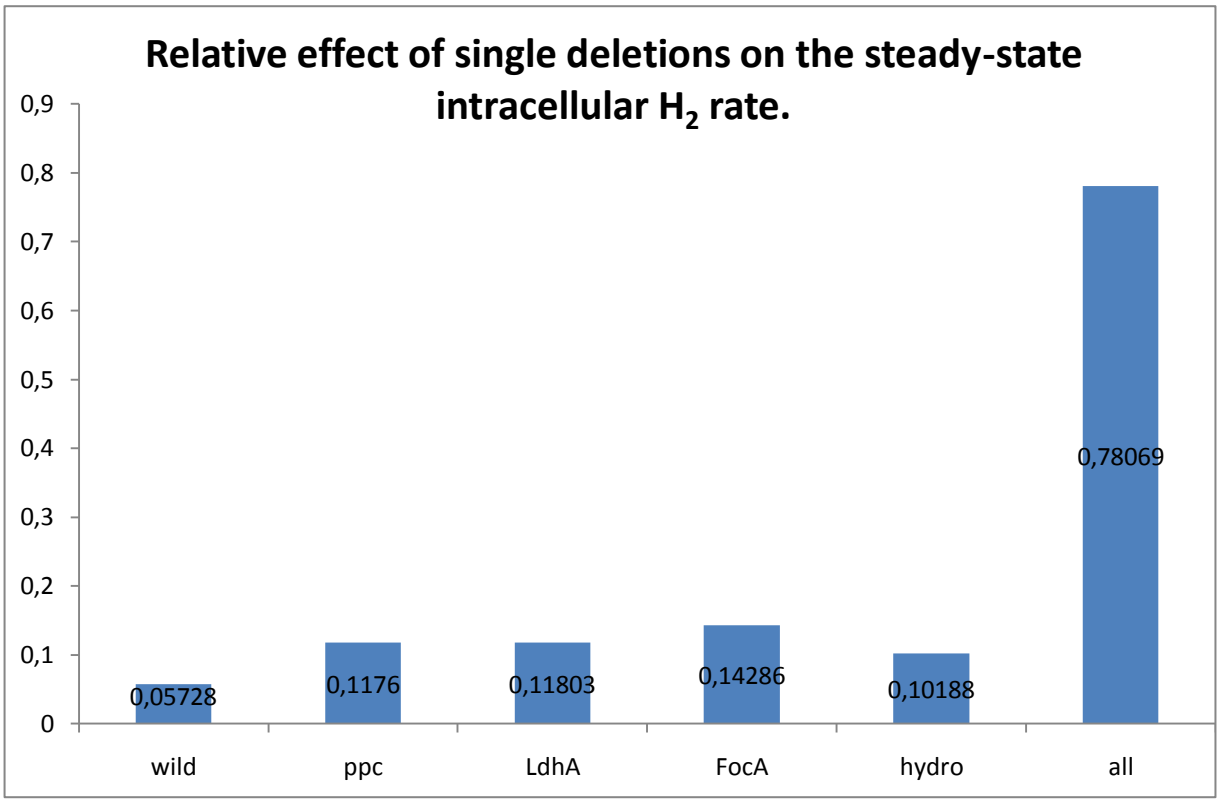


Diagram 1. Only one deletion in the mixed acid fermentation pathway can double its output. We can observe the major effect of the FocA deletion.

We noticed that for double deletions, the results cluster in three increased levels of production (cf. Diagram 2). A deletion with particularly strong associated effect: FocA. LdhA and PPC are middle effect deletions, whereas the deletion of both hydrogenases seems to have a lesser effect on productivity. Indeed, the combinations including FocA are always in the two higher levels of production, while those including the hydrogenases are always in the two lowest levels.

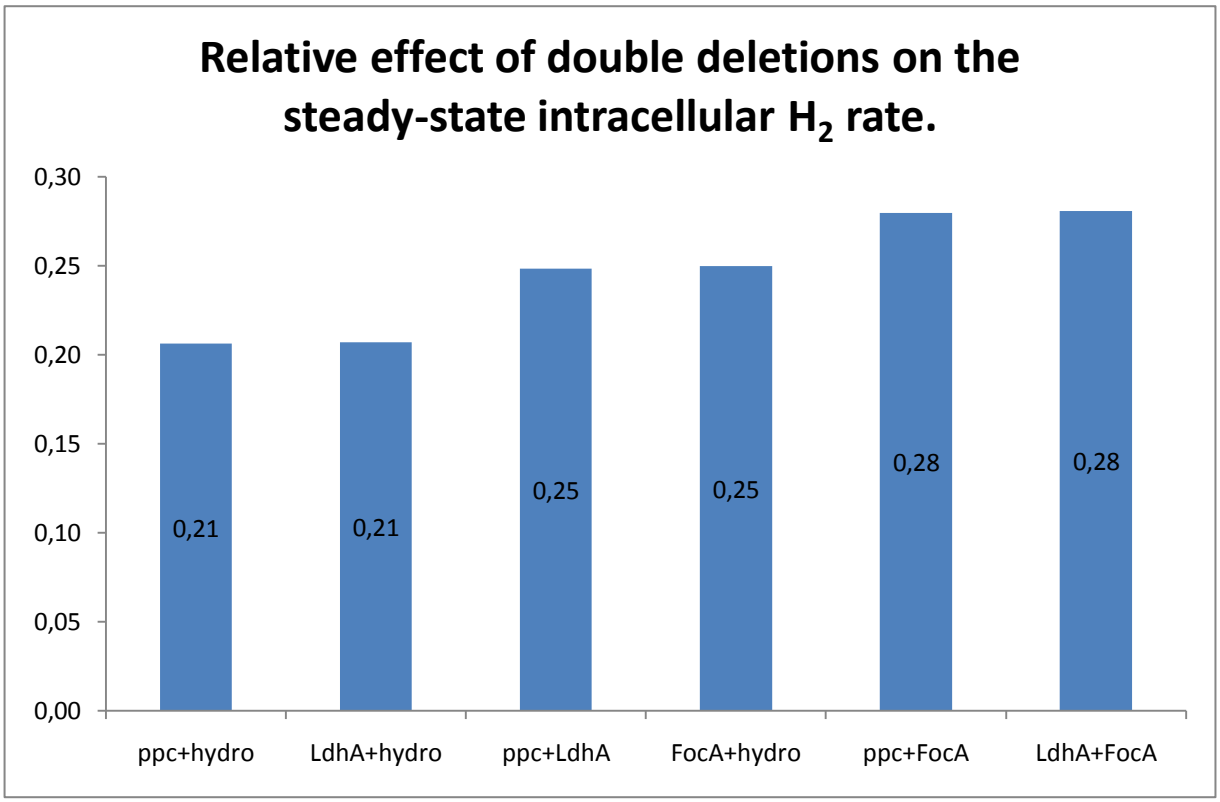


Diagram 2. We immediately notice the clustering in three levels of production, with the double deletion including FocA having a much greater influence on the dihydrogen rate than those including the both hydrogenases.

In triple deletions, the above results are confirmed and show even more the importance of FocA in dihydrogen production, while the weakness of the hydrogenases deletion seems less important. Indeed, we notice here two very marked dihydrogen production levels, one being for bacteria missing FocA and two other genes (no matter which) and the other one for bacteria with a functional FocA.

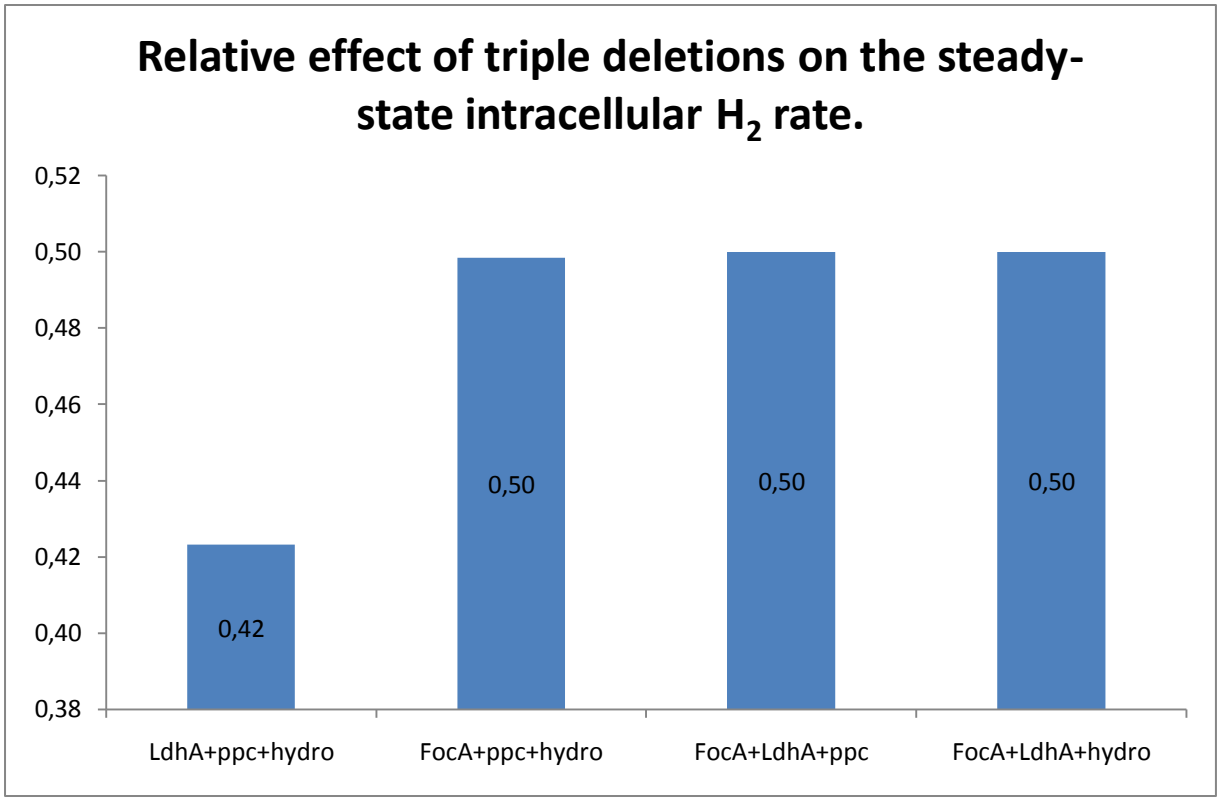


Diagram 3.FocA deletion seems prevalent in the effect of the dihydrogen production, whereas the hydrogenases seem poor targets for the deletion. We can observe that the triple deletions increase dihydrogen production up to 9 times compared to the wild type bacteria.

There is an important clarification to make here. The intracellular concentration in dihydrogen represented here are in no case absolute values, they are given in arbitrary units. Generally speaking, all of the numbers presented in this section are only for a comparative purpose. This is easily understood if we remember the largely empirical way to establish the parameters of the differential equations.

V.2 CELL DEATH MODULE

2.A QUESTIONS ASKED BY THE WETLAB

Here, after an initial modeling to theoretically validate the chosen system, we had to answer more specific questions, and with an immediate application : it was to choose the relative and absolute strength for the Ribosome Binding Sites (RBS) upstream of the genes used in this module, and to model the possible influence of the sequence the genes are built into.

To obtain a functioning module, we wanted the system created by the interactions of both plasmids not to compromise the long-term population density. And, in the event of a tenfold dilution, for the density of bacteria to immediately fall with no new bacteria growth, this would model the escape of our modified bacteria in the river.

2.B INITIAL EQUATIONS AND FURTHER ENHANCEMENTS.

When we have, like in this model, a certain complexity in the genetic regulations and we do model them, we generally use Hill terms, in addition to Michaëlis-Menten terms. Hill terms are non-linear, directly derived from Michaëlis-Menten like laws. They are proportional to C^n/C^n+K^n with n>=1 and where C is the concentration of the reactive and K in a constant.

To determine which order of the Hill terms to choose, we used Hill term order equals the number of effective regulations levels. This is because we see at each regulation level, proteins characterized by a Michaëlis-Menten type kinetic. If there are two regulations levels, we can reasonably assume that the saturation effects combine.

In a simple model, like the one of the dihydrogen, we can approximate that the amount of transcribed genes equals the amount of traduced protein, thus the order equal one.

It’s interesting to note that the order of the Hill’s term is representative of the complexity degree of the dynamics of a system; also, terms with an order equal or superior to two often have complex stability diagrams, including bistability phenomenon.

This model is richer in regulations level, since we consider the details of genetic expression and its regulation. We cannot posit anymore that the amount of ARN is constant, and we have to take into account this variability of transcription, the complexity of this ARN stage,

with the cooperative effects of the ARN-polymerase complex, we attribute a second order to the Hill terms put for the creation and consumption steps of the proteins we model. The first equation is more specific, since it models the bacterial density. This density classically increase, with an exponential phase followed by saturation of the system, hence the non-linear term of the logarithmic growth. The population, in addition to that density-limited growth, is also killed by the poison, proportionally to the bacterial density.

$$\frac{dN}{dt} = v_a.N(1 - \frac{N}{K1}) - vb.N.PARE$$

The first term is the natural growth tendency of a bacterial population.

The second term is the cell death term due to the poison.

$$\frac{d(HSL)}{dt} = v_c.N\frac{HSL}{HSL + K2} - v_d.HSLA.HSL - v_e.HSL$$

The first term express the fact that the signal-molecule HSL (cf. the Cell death module in Wetlab part, for a more detailed introduction to the role of signal molecules and a characterization of HSL) induce his own expression (it's here a typical induction, which obeys, too, to the saturability of substrates law), and that this is modulated by the bacterial density: the more bacteria we have, the more probable will be the entry of HSL in the cell.

The second term is a degradation term of HSL by the specific enzyme; the third one is a non-specific degradation or diffusion term.

$$\frac{d(HSLA)}{dt} = v_f\frac{K3}{HSL + K3} - v_g.HSLA$$

The first term express the inhibition by HSL of the promoter which controls the gene coding for the HSLase.

The second term is a non-specific degradation term.

$$\frac{d(PARE)}{dt} = v_h\frac{K3}{HSL + K3} - v_k.PARE.PARD - v_i.PARE$$

The first term is identical to the one of the above equation, although with a different coefficient, because of the possibility of a different strength RBS.

The second term expresses the mutual neutralization of the antidote and poison, by complex formation.

The third term is a non-specific degradation term.

$$\frac{d(PARD)}{dt} = v_j \frac{HSL}{HSL + K2} - v_k.PARE.PARD - v_i.PARD$$

The first term express the activation by HSL of the promoter, just as in the HSL equation but with a different coefficient due to RBS.

In a second time, to refine the system, we also chose to better take into account the subtleties of the culture medium, in which we add HSL in growth conditions, by clearly separating the two co-activators (or inhibitors) of the promoter. It was also to add equations, permitting to better take into account the formation of complexes, important in this system, since these complexes activate or inhibit the whole system. We again explicitly made the difference between the concentration in ARN and in protein.

Finally, we have distinguished the intra and extra-cellular HSL, to better take into account the HSL massive outflow phenomenon, by diffusion, during the dilution. This will allow our model to predict a possible peak of HSL, causing a partial stability (and even a slight initial growth) of the population, immediately after the dilution.

Here are the final equations:

$$\frac{dN}{dt} = v_a.N(1 - \frac{N}{K1}) - vb.N.PARE$$

$$\frac{d(LUXR)}{dt} = r_2.v_1 \frac{LUXRHSL^h}{LUXRHSL^h + K1^h} - v_r.LUXR - v_c.LUXR.HSL + v_d.LUXRHSL$$

LUXR is at the same time the transcript of the gene and the corresponding protein: the coactivator (or inhibitor) with HSL of the promoters. R2 is a coefficient put to model the relative strength of the RBS. LUXR is under control of the promoter activated by the LUXR-HSL complex, hence the creation term, and we notice the second order of the Hill term. The second term is a non-specific degradation term and the last one comes from the dissociation of the complex.

$$\frac{d(LUXI)}{dt} = r_1.v_1 \frac{LUXRHSL^h}{LUXRHSL^h + K1^h} - v_p.LUXI$$

LUXI designates the gene coding for HSL. We notice again the coefficient of the RBS and the activation by the complex, together with the non-specific degradation term.

$$\frac{d(HSL)}{dt} = k_s.LUXI - v_h.HSL.HSLA - k_i.HSL + fke.HSLe$$

HSL is translated using the LUXI transcript, hence the first term. The second one is a degradation term by the HSLase, proportional to this enzyme and to HSL. The third term is a loss term due to diffusion outside of the cell and the last one symbolizes the reverse phenomenon: the entry of extra-cellular HSL. Fke is a function of N, it allows us to model that, the more dilute the population is, the more HSL will leave the cells by passive diffusion. This will explain the massive outflow of HSL observed at the very first stages following an important dilution.

$$\frac{d(HSLe)}{dt} = k_i HSL - fke.HSLe - v_z.HSLe$$

HSLe (extra-cellular) is modulated by the diffusion in and out of bacteria. The last term is a loss term: when HSLe is too far away, it has no influence on the system anymore.

$$\frac{d(LUXHSL)}{dt} = v_c.LUXR.HSL - v_d.LUXHSL - v_q.LUXRHSL$$

LUXRHSL represents the complex formed by these two signal molecules. Its concentration is proportional to the one of its constitutive parts, minus the quantity of complex that dissociates and a non-specific loss term.

$$\frac{d(HSLA)}{dt} = r_4.v_2 \frac{K_2^m}{K_2^m + LUXRHSL^m} - v_w.HSLA$$

Once again, we can see a coefficient put for the RBS. Here, the complex formed by the signal molecules inhibits the activity of the HSLase, which is under control of the poison-promoter. There is also a non-specific degradation term. .

$$\frac{d(PARD)}{dt} = r_3.v_1 \frac{LUXRHSL^h}{K1^h + LUXRHSL^h} - v_k.PARD.PARE + v_m.PARDPARE - v_i.PARD$$

PARD is the antidote, thus under control of the promoter activated by the signal-complex, hence the first term. The next one is due to the complex formation of PARD with the poison, and the third one to the dissociation of the complex. The last term is of non-specific loss.

$$\frac{d(PARE)}{dt} = r_5.v_2\frac{K2}{K2 + LUXRHSL} - v_k.PARD.PARE + v_m.PARDPARE - v_i.PARD$$

PARE is the poison, so that it's inhibited by the signal-complex. The second and third terms are identical to those of PARD, and the last one is a non-specific loss.

$$\frac{d(PARDPARE)}{dt} = v_k.PARD.PARE - v_m.PARDPARE - v_n.PARDPARE$$

The antidote-poison complex is formed from these two molecules and degraded by their dissociation. There is also a non-specific degradation term.

RESULTS

Before we tried to answer the questions asked by the wetlab part, we tried to establish the different values of the parameters (and the order of the Hill's terms) allowing a satisfying modeling of the module, and thus mathematically validating the chosen system. These results will be introduced at the end of this section, because they are a summary of the modeled behavior of the bacterial population, but we have to keep in mind that this stage is the very first one.

The first question answered a practical interest, because the main goal was to model the ideal relative value of RBS for each genes carried by plasmids. So we first simulated various configurations in this way.
We managed to vary the values of coefficients used in the above equations, which symbolized coded proteins on genes.
We studied the behavior of our population before and after a ten-order dilution as we were varying the RBS relative values (one compared to each other). All parameters that gave an unstable population in the reactor or, to the contrary, stable or increasing population after dilution were immediately rejected.

Here are the main results obtained during our simulations:

Note that:
- According to the equations r1, r2, r3 represent the strength of "antidote" RBS and r4, r5

represent the strength of "poison" RBS.
- r1 represents LUXI RBS, r2 represents LUXR RBS, r3 represents PARD RBS, r4 represents HSLase RBS, r5 represents PARE RBS.
- We estimate that 6 units of time on the graphics represent 1 hour. We obtained this estimation by looking at the time a population needs to stabilize itself.

Step 1:
Vary R=r1=r2=r3=r4=r5

In the reactor:
For small values of R (<1 "relatively") the population collapses drastically after 5 or 6 hours spent in the reactor.
For higher values of R (>1) the population stabilizes itself. The bigger the value, the higher the population stabilizes itself.

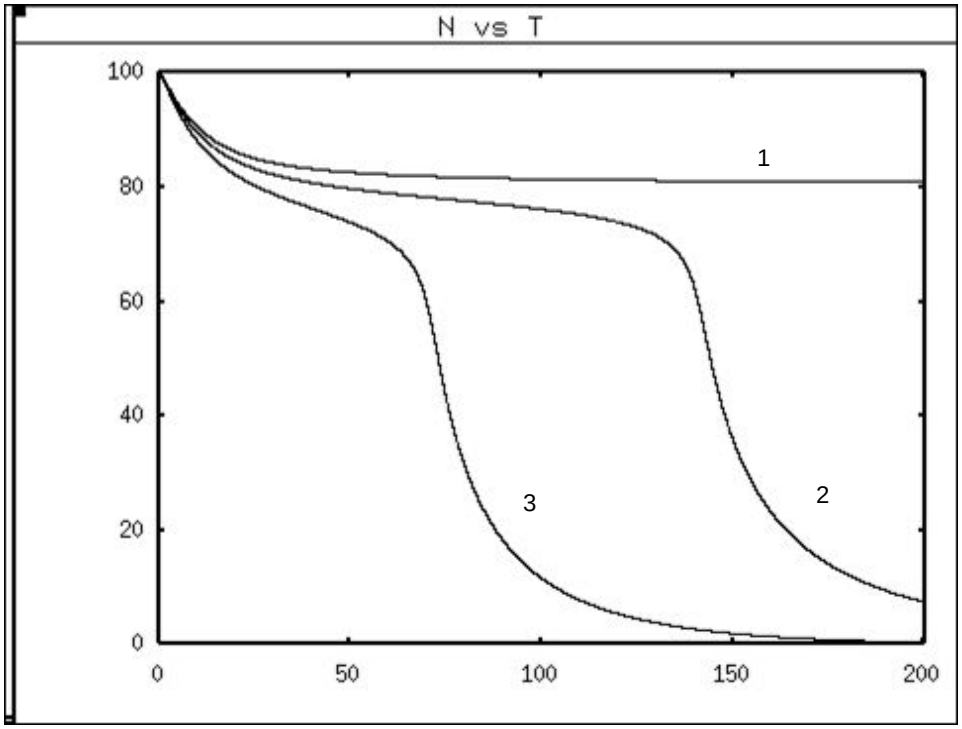
In the river:
For small values of R (<1) the population is stable, which is a situation to absolutely avoid.
For higher values of R, we first observe a "baby-boom" quickly followed by a fall of population. (The importance of both of these phenomenons is proportional to R.)

A first result is obvious: we will have to work with powerful RBS.

Step 2:
Vary the poison (r4 = r5) and the antidote (r1 = r2 = r3) separately.
According to previous results, we will only use high values of RBS. (>1 "relatively")

Step 2.1 Improve the poison/antidote ratio

Results in the reactor:
- For a ratio of 1.05 (poison 5% stronger than antidote): during the 24 first hours, we only loose 5% of the population (compare to a ratio of 1), but later, the population does not stabilize itself.
- For a ratio of 1.1 (poison 10% stronger than antidote): during the 24 first hours, we loose 15% of the population (compare to a ratio of 1), and after 24h the population collapses.
- For a bigger ratio, the population collapses too quickly.
cf picture 1



Picture 1: Evolution of the population density in the reactor for different values of the ratio poison/antidote: 1,05 ; 1,1 and 1,3. Respectively curves 1, 2 and 3. N=population density and T=time.

Results in the river:
The population falls down more quickly when the ratio increases, but the difference is not that interesting.

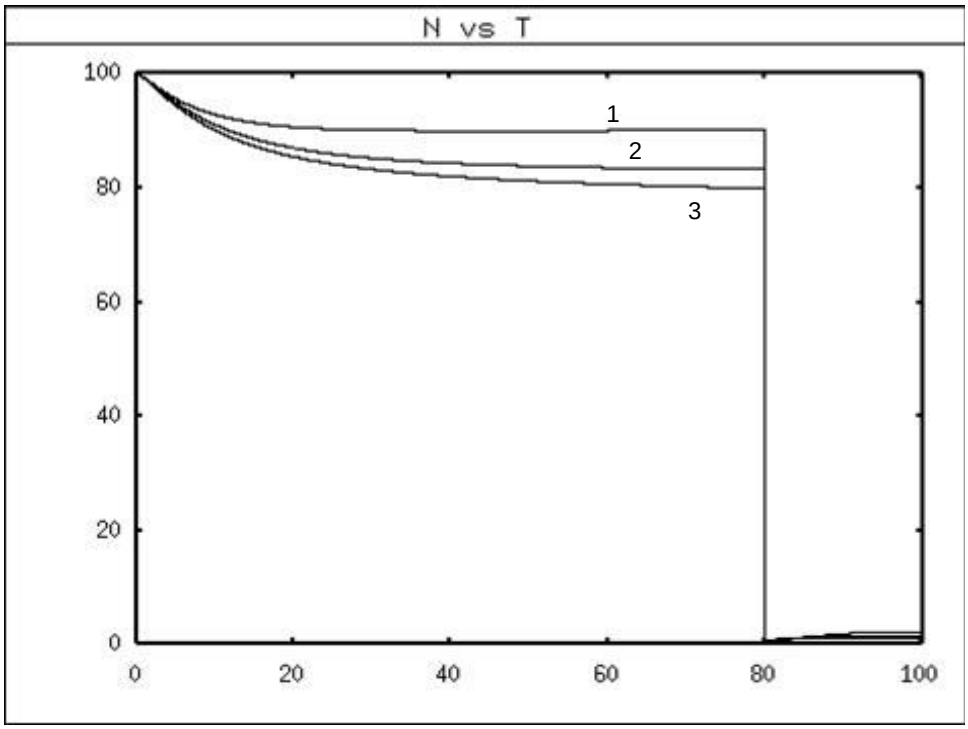
Step 2.2 Improve the antidote/poison ratio

Results in the reactor:
- Increasing the ratio until 1.5 enables the population to reach a stable state at 92% of its initial situation. Compare to 85% for a ratio of 1. The bigger the ratio, the higher the population will stabilize itself.

Results in the river:
- If the ratio is over 1.5 the population will not decrease any more.
- It is better that we do not go over a ratio of 1.2 in order to avoid a huge baby-boom (inevitable if the ratio is bigger than 1).

Conclusion for step 2:

In the reactor:
If we increase the poison by 10% (max), it does not have significant effect while t<24h.
If we increase the antidote by 20%, the population stabilizes itself at 90% of its initial situation. Compare to 85% for a ratio of 1 . As shown on picture 2.



Picture 2: evolution of the population density for 3 different values of the ratio antidote/poison : 0,9 ; 1 and 1.2. Respectively curves 1, 2, 3. N=population density and T=time.

In the river:
If we stand in a bracket of +10% poison/+20% antidote, our population collapses in a significant way.

Step 3 Vary antidote ones compare to each other, and similarly for poisons.

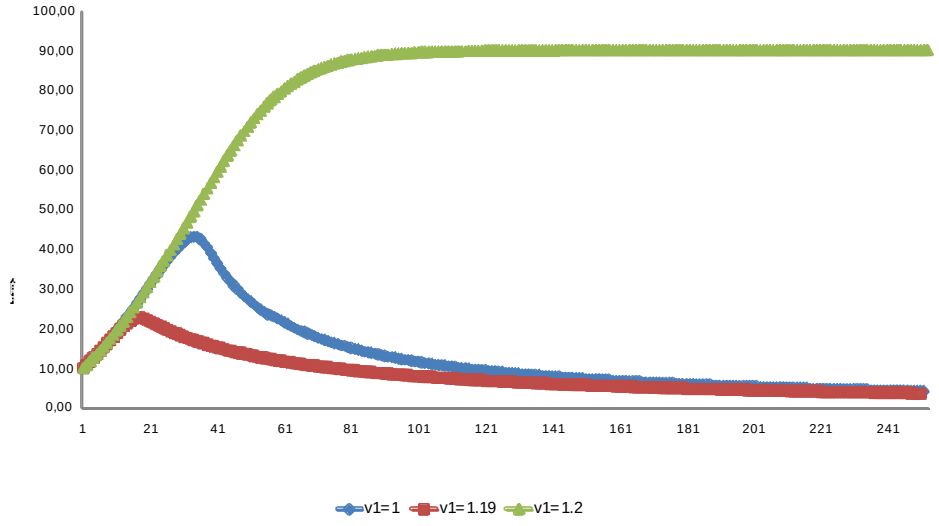
If we keep in mind the last results (rules) we obtained (mainly, r>1 and the fact that ratio antidote/poison stands between 0.9 and 1.2),
the effects on the population are not that different from the previous cases, whatever the configuration is. So we can keep r1=r2=r3 and r4=r5

Our recommendations to the wetlab part were thus simple, design the genes with RBS of strength 1 (practically speaking, it's hard to find strong RBS which are only a little bit different). We then could start a more analytic part of our work.

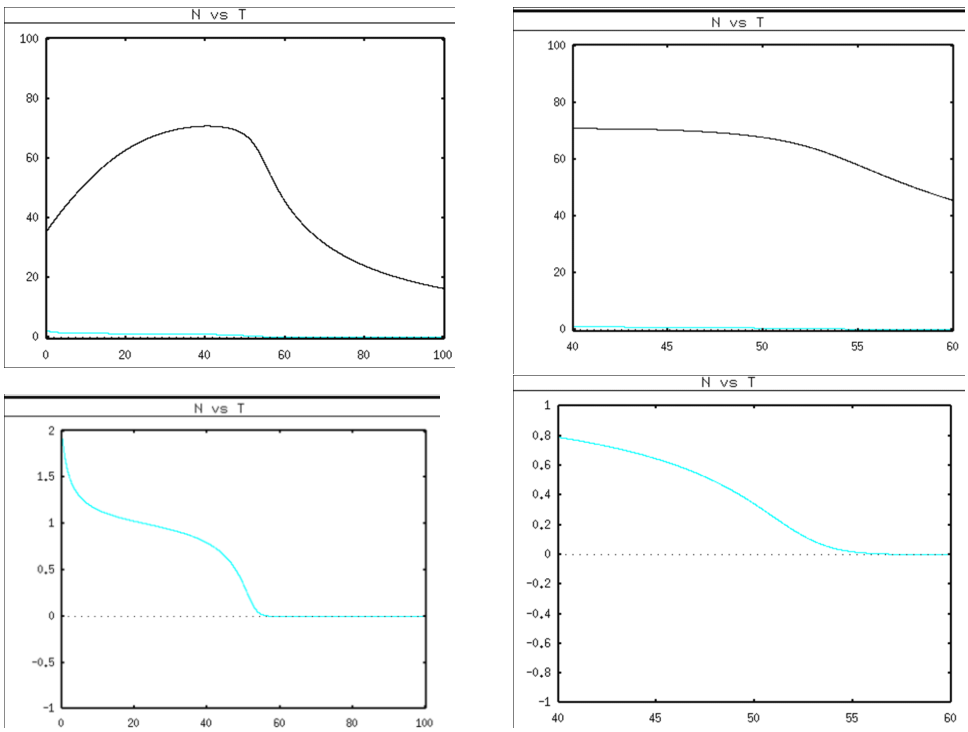
Here, because of the order of Hill terms strictly upper than one in many equations of the module, we were hoping for special stability situations to occur and we tried to establish stability diagrams of the system.

V1 is the key parameter of the antidote plasmid, since it's the coefficient standing for the transcription speed of the system. We generally have, in the absence of regulation systems or of special RBS value, arbitrarily attribute the value 1 to each parameter of that type. With this value v1=1, the population diluted 10 times collapses very fast. We then increased step-by-step that value of v1 to observe which value will lead to the stability of the population and if there exist intermediate population densities between stability and collapse of the population.

The result is a very strong step effect. Indeed, either the population remains up to 80% of its initial density, or it collapses completely. This is very clearly shown on graph1, where the curve in red and blue respectively represent the evolution of the population for the values of v1=1 and v1=1.19, when the green curve represents the evolution of the population over time if v1=1.2.

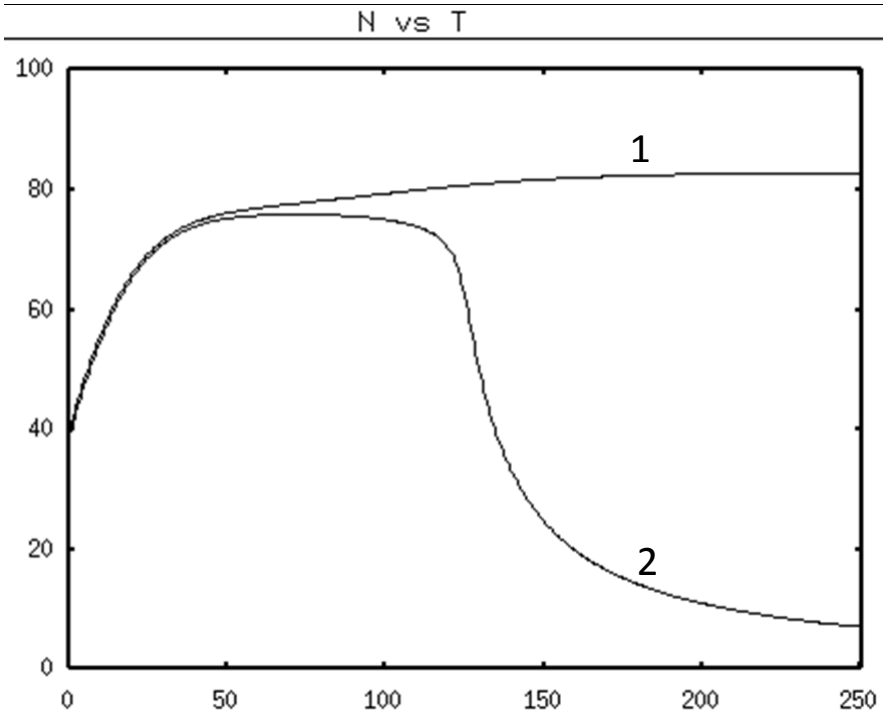


Graph 1. Note the very strong step effect between the values of v1 1.19 and 1.20, and the fact that population does not collapse immediately, due to the initial HSL concentration.



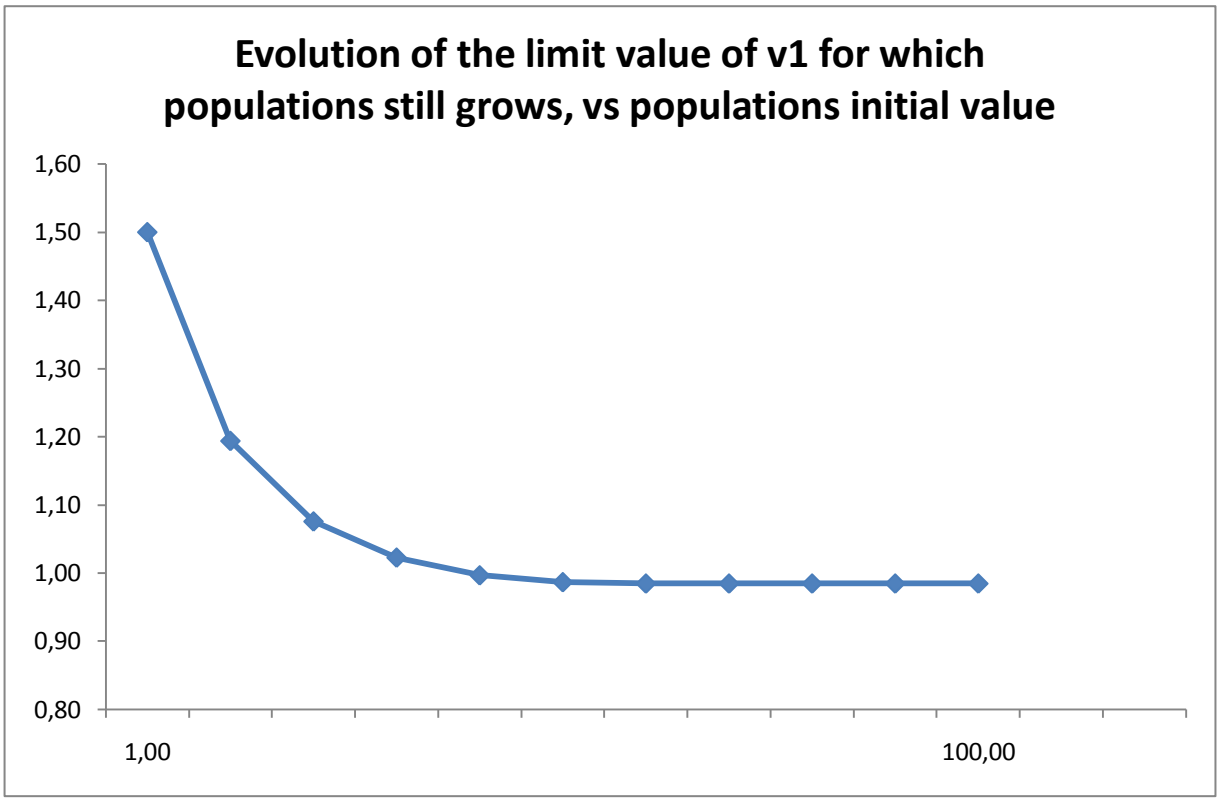
Graph 2. In black, the curve of population density (N) against time; in blue, HSL concentration against time, there is a very strong correlation between these two curves.

In the same way, we fixed the value of v1 to 1 and studied the behavior of the population depending of its initial density. We can see (picture3) that for Ninitial=39, the population grows and stabilizes around 80%, when for Ninitial=38, this population density collapses. We still notice that the bacterial density, few instants after the beginning of the simulation, grows in a similar way to what happens for Ninitial=39. This is explained by the non-nul initial concentration of HSL which maintain the population even if the quorum sensing isn't sufficient to produce it during some time (see graph for the parallel between concentration in bacteria and HSL). We chose to give a non-null starting value to the HSL concentration because some is added in the initial growth conditions and, if some bacteria escape in the river, which is similar to a dilution, their intracellular rate of HSL won't be null. However, we can emphasize an important step effect and a bistability phenomenon.



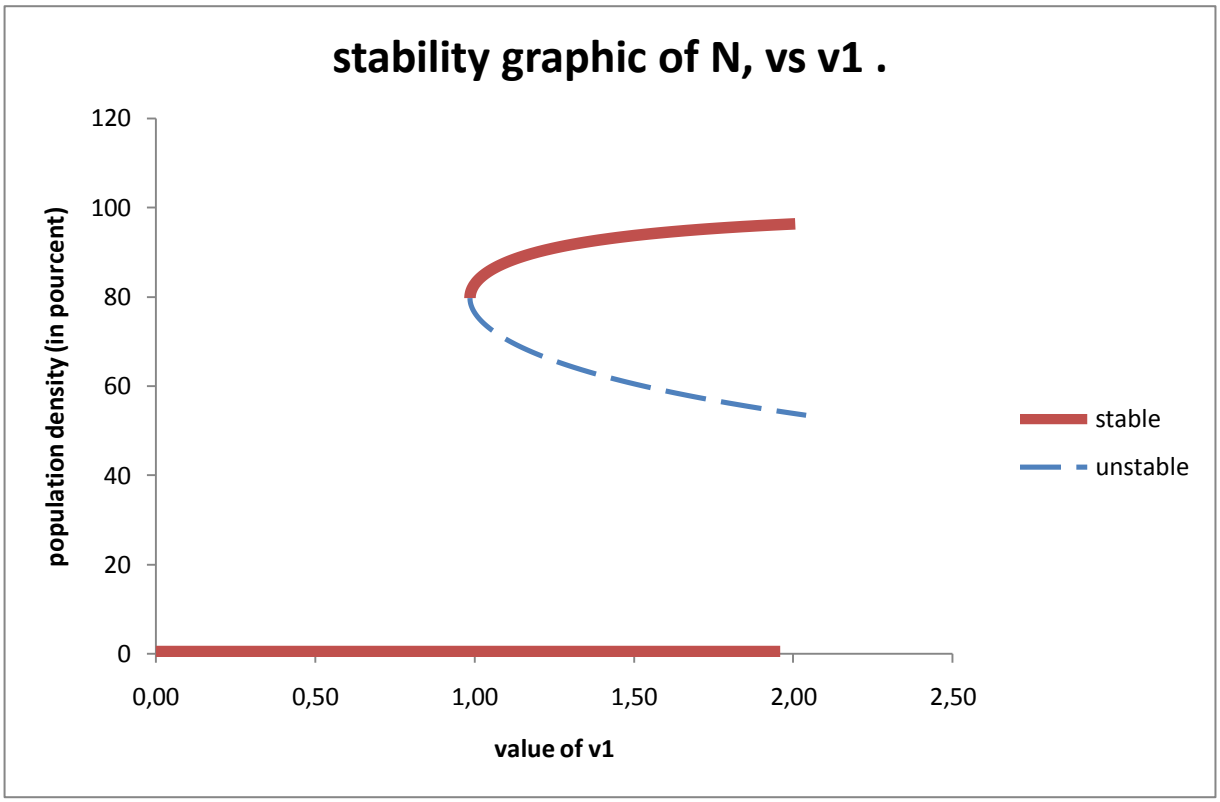
Picture 3. That's the step effect which relates to initial cell density. The first curve is of an initial density of 39% and the second has an initial density of 38%. In both situations, populations do not immediately collapse.

This step effect is studied once more in the next graph (graph3) which shows the evolution of the minimal relative transcription speed of the antidote plasmid (v1) such that the population is stable, depending on the initial population density. We logically see the decrease of the v1 value when the initial density increases, but more unexpected, we notice a limit value of v1 (=0.985) such that, no matter how big the initial population is, this population finally collapses. This emphasizes the great importance not to use a too small value of RBS for this plasmid, to avoid the collapse of the bacterial population in the reactor.



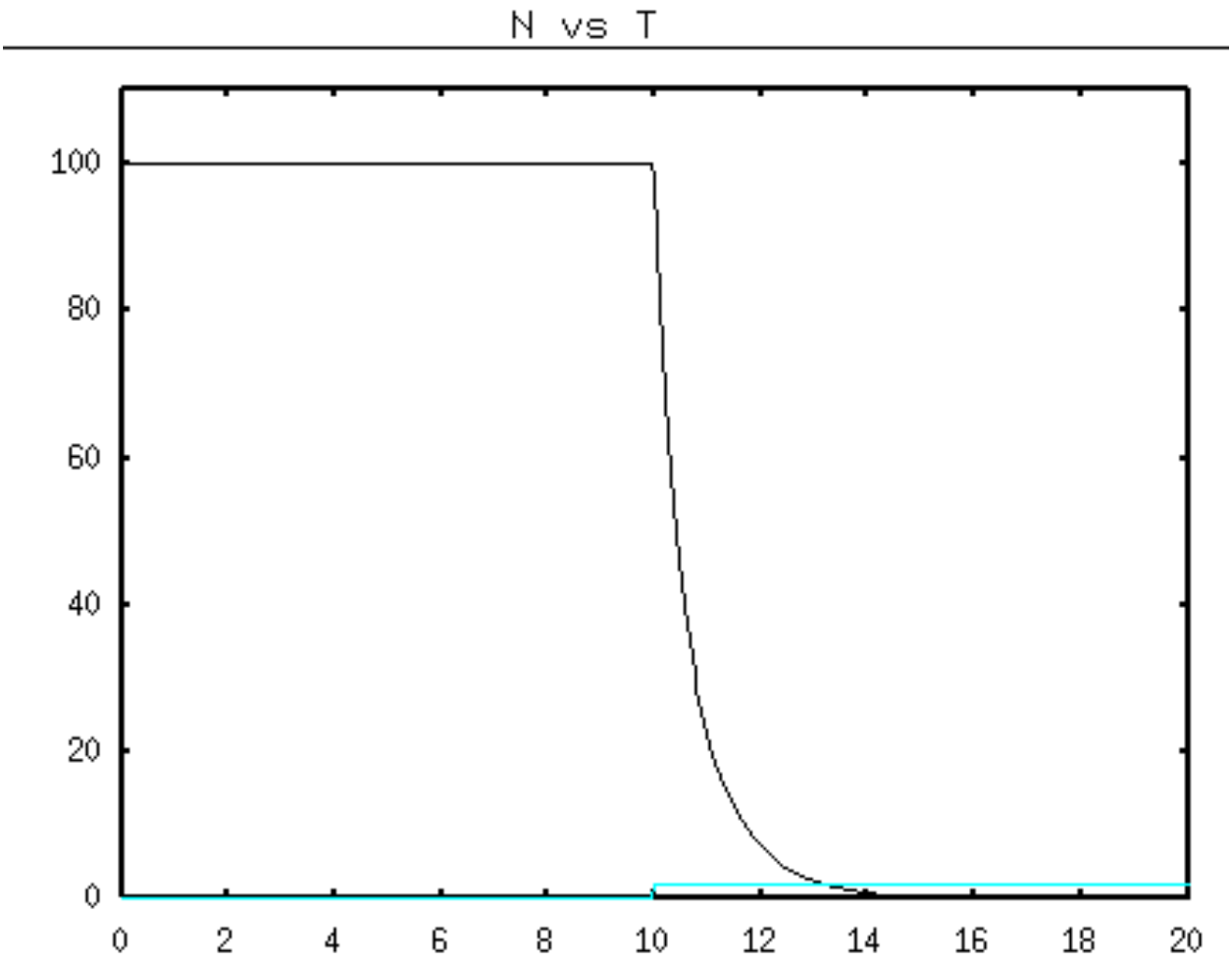
Graph 3. At the beginning, a small increase in initial density causes the needed value of v1 to rapidly decrease, then the curve stabilizes and is eventually constant at v1=0,985, for population density value of 60 and more.

Then, using the data computed by the auto software, we draw the stability graph for N (population density, in % of the maximal value in reactor; this symbol will be used in all this section) for different values of v1 (graph4). We see here two disjoint stable curves; the first one, which is a constant equal to zero is the only one for v1<0.985, as expected, and continues for any value of v1. This is explained by the fact that, whatever the value of v1, it's always possible to find a Ninitial such that the population collapses. The second curve only starts at v1=0.985, and the equilibrium value of N increases with v1. This means that, whatever the initial value of the population density, if it's bigger that the step value, the equilibrium value of N will be the same, for a given v1.

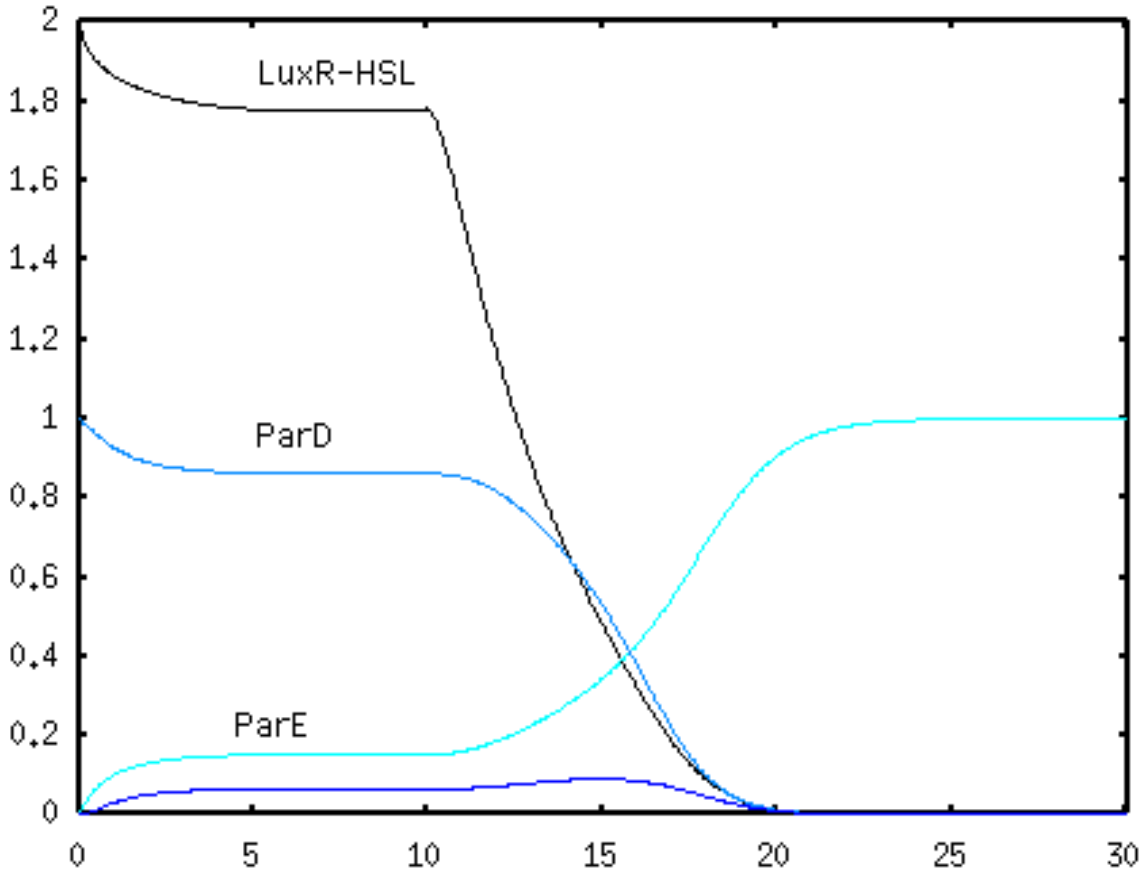


Graph 4. Stability graph of N vs v1. Note the presence of two stable curves for a same value of v1: that's bistability.

Finally we did a little simulation of a real situation, with a population in the reactor initially equal to 100% and a dilution factor of ten, at t=10. The first graph (picture4) is quite clear: during the dilution, the density falls below the critical value, and the population then self-destructs. Zooming on different variables for the population (picture5) we notice the decrease in "growth" proteins (HSL, ParD, LuxR-HSL) and the increase in « destructive » proteins (HSLase, ParE), from the dilution time through a compensation point around 17, in which values are inverted which is the point of no return for the population and until there are no more "growth" proteins. The system chosen for the programmed cell death thus seems to work perfectly well.



Picture 4. Population densities change, before and after the dilution step at t=10. See that, as wanted, the population collapses just after the dilution step, and that it doesn't grow again.



Picture 5. Change of some intra-cellular compounds before and after a dilution step at t=10. Note the inversion of the values of ParD and ParE, and the collapse of the activation complex LuxR-HSL.

VI. THE EXTRA-SCIENTIFIC ISSUES OF HYDROCOLI

VI.1 INTRODUCTION

The object of this paper is not to add to the scientific contributions of our colleagues, but to provide an accessible overview of the type of question that the IGEM competition raises, all the while focusing specifically on the issues surrounding the ULB team's project. We believe that if synthetic biology is to become a part of our daily lives, it is necessary to expand research to questions which are raised by this field but cannot be answered within this field. In this way we hope new perspectives can be explored outside of the usual and often restrictive frame of reference of "hard" science.

The *hydrocoli* strain that the ULB Brussels team has been working on for IGEM is designed to produce energy from waste. Specifically, the research concerns the production of *hydrogen* (in exploitable quantities) from *wastewater*. If the project is successful, Jules Verne's "Coal of the future" may well be water, not as a composite body, but as the vehicle of the components from which hydrogen can be harvested.

This project has far reaching implications. The fact that it focuses on the treatment of wastewaters and the production of clean energy means that it has potential repercussions which go above and beyond the scientific merit of the challenge. This is why a more pluridisciplinary approach seemed necessary.

This paper, which will examine the non-scientific issues surrounding the project, is made up of five parts. We begin with an examination of the ethical and philosophical questions raised by *hydrocoli*. This first chapter includes a brief explanation of the project in laymen's terms: as it was written by philosophy and economics students, we wanted it to be accessible to our peers. Moreover, it is important to render the content of this type of competition accessible to anyone who is interested, particularly as biotechnologies concern everyone. The chapter on ethics also includes a cursory overview of the possibilities for humanitarian aid. This is developed more in depth in the fifth chapter, which is concerned with the possible economic consequences of its implementation. Chapter 2 concerns a pragmatic aspect of ethics, bio-safety, and includes a brief overview of the relevant legislation. Chapter 3 examines issues of property rights. Property rights, as a field which concerns both ethics and economics, makes an ideal segue into chapter 4, which examines the marketing of *hydrocoli* and of the products of biotechnology in general. The last chapter, as we mentioned, explores the economic possibilities of the implementation of *hydrocoli.*

For the purposes of this paper, we consider all the potential ramifications of the project, including those which may not be an issue in the immediate future. The importance of this approach needs no explanation in the wake if such ecological catastrophes as the hole in the ozone layer. As the saying has it, “better safe than sorry”. Besides the ethical reasons for this approach, the importance of taking into consideration every conceivable consequence, be it a question of an ethical, philosophical, economic, social or ecological nature, cannot be overstated if we hope for a successful use of *hydrocoli* in the future. A complete analysis of the project is thus a useful tool for our team if we hope to see our research applied one day.

VI.2 ETHICAL AND PHILOSOPHICAL ASPECTS OF THE IMPLEMENTATION OF *HYDROCOLI*

" Oui, mes amis, je crois que l'eau sera employée un jour comme combustible, que l'hydrogène et l'oxygène qui la composent, utilisés ensemble ou séparément, fourniront une source inépuisable de chaleur et de lumière, et d'une intensité que la houille ne saurait avoir. Ainsi donc, rien à craindre... L'eau est le charbon de l'avenir.” Jules Verne, L’île mystérieuse, 1873.

This first chapter explores some of the ethical and philosophical aspects of *hydrocoli.* It begins with a popularized explanation of the project, aimed at the reader with no scientific background. We will then go on to explore the possible applications in terms of humanitarian aid and energy production. Lastly, we will take a more purely philosophical approach, exploring the implications of biotechnologies for Man’s place in the world through a heuristic approach.

In order for the reader to understand the full implications of certain technicalities, it is essential to introduce a minimum of scientific lingo. Three of the technical aspects of the project bear mentioning for the purposes of this paper:

1) The use of *Escherichia coli*[6] as the bacterial base.
2) The fact that this new strain of E coli will aim at providing a complete water treatment process, through the inclusion of several modules, such as cellular death or heavy metal detection.
3) The association of wastewater treatment with hydrogen[7] production.

[6] See Box 1

[7] See Box 2

These three aspects are important for several reasons:

Firstly, because the use of E. coli as a base makes our modified strain easy to use and to improve upon: E. coli is the best-known and most commonly used bacterium in laboratory research.

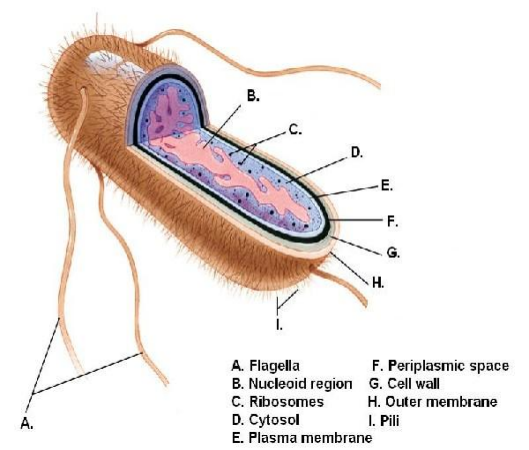
Secondly, developing and exploiting this bacterium is not costly in terms of time or money because the technology needed is not particularly sophisticated. This is an important advantage for *hydrocoli* when compared with other water treatment processes.

Thirdly, the fact that, through synthetic biology, E. coli can be modified to include new characteristics is a non-negligible advantage. This makes it possible to transform E. coli into a strain capable of breaking down and neutralizing most matters commonly found in wastewater.

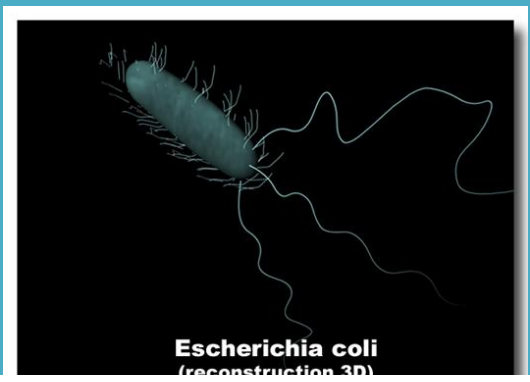
Box 1

** Escherichia coli:*

E Coli is originally present in the digestive systems of mammals .In people it makes up 80% of the aerobic flora. There are many different strains of this bacteria, some dangerous.



At this stage, E Coli is the most closely studied living organism because it was discovered so long ago (in 1835 by Th. Escherich) and because its culture is so easy: cellular division takes place every 20 minutes at 37°. The genetic makeup of non-pathogenetic laboratory E Coli was entirely sequenced by 1997. These characteristics make this bacterium the perfect tool for research in molecular biology.



Your run-of-the-mill strain of E. coli can break down certain matters that are commonly found in waste. However, the modified strain will be able to break down organic matters such as cellulose, signify the presence of heavy metals[8] (perhaps even denitrify its environment[9]) and even eliminate methanogeneous bacteria which would otherwise curb the production of hydrogen by consuming said hydrogen through fermentation. Having said this, if we are to follow the precautionary principle[10], it is safer to repress the methanogenous bacteria than to destroy them. In this case, following the precautionary principle means preserving biodiversity in the environment surrounding the water treatment facility. In fact, certain bacteria which we could inadvertently destroy could be essential components of the eco-system at the exit of the water treatment facility.

If we were to opt for the elimination of the methanogenous bacteria, 10% of the water would have to be left in its natural state in order to preserve the biodiversity of the water source at the exit of the utility. Either way, precautions must be taken to preserve biodiversity because, as well as being an essential ethical principle, the preservation of biodiversity is a legal imperative.

The destruction of the E. coli strain after it has completed its tasks can be integrated as an automatic fail-safe into the bacterium itself, increasing its practicality and avoiding contamination risks. The risk of polluting the outside environment and modifying the eco-system

Hydrogen and its many advantages:

- *The hydrogen atom is one of the most abundant on Earth.*
- *Dihydrogenous combustion is very energetic (120MJ/kg, or 2, 2 times more than natural gas. 1kg H2 = 33, 33 KWh = 120 MJ = 2,855 kg petroleum equivalent).*
- *Non-polluting, non-toxic, and its combustion creates no carbon dioxide.*
- *It's not heavy, which makes it safer because it diffuses quickly, avoiding the risk of explosion.*
- *It can be used in combustion batteries and electrolysis, and thus can be used with one of the major energy vehicles such as electricity.*

[8] The detection of heavy metals is prefered to bioaccumulation because it presents less environmental risk. Heavy metals which remain in the fertiliser resulting from the process can cause contamination problems.

[9] This denitrification is theoretically possible but needs several metabolic paths, making it difficult in practice.

[10] The precautionary principle was originally a philosophical concept but became a part of legislation in many countries. French law (1995) defines it as follows: in the absence of certainty… effective and appropriate measures which avoid serious irreversible damage must be possible at a reasonable economic cost.

are thus practically non-existent thanks to the poison-antidote system which the engineers are attempting to put into place in the cellular-death module.

The modification of E. coli in such a way as to give it the ability to fulfill several functions presents real advantages when compared to traditional water purification methods. Traditional methods require several different bacteria that must be monitored in a purification station. This is clearly a more complex, and therefore more costly, method.

Lastly, this strain is also modified in such a way as to produce hydrogen while fulfilling its other tasks. The production of hydrogen will be integrated into the water treatment system and it would be produced using the elements broken down from the waste present in the water. Moreover, synthetic biology actually makes it possible to increase the strain's production capacity (by deactivation and over expression of certain genes) making it a possible cost-effective alternative to current energy generation methods. At this point, the team lacks sufficient information concerning energy production rates but it is very likely that the energy produced will at least be sufficient to run the treatment facility. Moreover, it is a very real possibility that the amount of hydrogen produced would exceed the amount necessary for a self-sustaining utility. The excess hydrogen could then be stored in sufficient quantities for domestic and/or industrial use. Furthermore, it should not be forgotten that even if the hydrogen production rates are small at first, our strain is a prototype that can be improved upon in the future. In any case, this project signifies real progress on an ecological and environmental front. No matter what the levels of hydrogen produced, any gain of energy in the process of waste treatment is in fact progress.

Hydrogen is non-polluting and its combustion emits only steam but this does not mean that hydrogen production techniques are environmentally sound. For an energy source to be truly clean, the production process itself must be clean. The most promising technologies are those which do not create any carbon monoxide or carbon dioxide because greenhouse gases are currently our most pressing concern. Our outlined method thus presents a serious advantage when compared to other methods because it creates no greenhouse gases at any point.

Hydrocoli is even more advantageous than most energy sources that fall under the heading "renewable". For example, the production of electricity through photovoltaic panels or windmills is less advantageous, qualitatively speaking, because the infrastructure relies on polluting or exhaustible resources, such as terbium, dysprosium and neodymium which are 'rare earths'[11]. When you add the fact that the maximum period of exploitation of these technologies is not sufficiently long to justify their energy costs, it is clear that they are not energy solutions for the long term. The aforementioned problems are non existent with

[11] See Box 3

technologies which rely on synthetic biology because it uses living elements, bacteria, which produce absolutely no waste and are exploitable in existing infrastructures.

Besides the advantages for energy production, *hydrocoli* could have positive consequences for water access: new filtering and purification facilities that run with technologies relying on synthetic biology could revolutionize humanitarian aid and development. Running water is so common in industrialized nations as to be taken frequently for granted, but in developing countries water is an extremely problematic issue, so much so that the United Nations made "the decrease by half of the percentage of the population without access to drinking water[12] or basic cleaning facilities" one of their urgent "goals for the Millennium[13]" to be reached by 2015. Furthermore, the issue of access to drinking water goes hand in hand with the issue of the purification of wastewater. In fact, contaminated water is responsible for the deaths of 5000 children per day (one death every twenty seconds) and ingested wastewater can cause diarrhea, making it is the main cause of mortality in children under five.

The ubiquitous and difficult nature of the wastewater treatment problem arises from its complexity. The fixed costs of water purification are huge, meaning developing areas do not have access to the kind of global water treatment means that are commonplace in the west. This is why the development of our simple water treatment technology could have a revolutionary effect on humanitarian aid.

Box 3 :

Rare earths

These are chemical metallic elements extracted from minerals. This category includes scandium, yttrium and 15 lanthanides. The elements have particular properties and are highly coveted in the alternative energy development field.

Terbium, dysprosium and neodymium are lanthanides used in hybrid cars, in wind turbine generators and in solar technology. Their demand is constantly growing. This poses a serious problem. It is estimated that terbium 65 stocks will be depleted in 2012. Moreover, mines where they are extracted are often extremely polluting.

[12] Access to water is defined as 20 liters per day and per person

[13] See Box 4

Indeed, the infrastructure needed is minimal, making this technology potentially cost-effective and easy to use for groups on a small scale, such as families. Water purity is a problem especially in warm weather areas, because that is where bacteria survive and breed. Our innovation in fact takes advantage of this fact, because the modified strain of E. coli is well-suited to just such areas.

Another major water contamination problem arises due to the absence of toilets. Once our technology is implemented, wastewater could be evacuated and treated on the spot even in the most isolated areas.

The difficulty of access to clean water is an important factor for economic development because the difficult task of finding water takes several hours a day. Generally the task falls to women and children, who may miss several hours of school fulfilling the chore.

Access to clean water is also absolutely essential in the fight against many diseases which can be simply eradicated if adequate wastewater facilities are provided. Currently, water purification tablets or filters are distributed by several NGO's but these methods don't have the added economic and social value of the implementation of a water utility and an integrated distribution circuit and are not a long term solution because reliance on foreign aid creates new problems.

The ideal end result of our team's project would be the easy installation of a water network, on any scale (village, city, country) through the implementation of small purification centers. The water purification would be linked to the production of electricity from hydrogen for areas that are cut off from the main energy networks. In this way and on a local scale, these utilities could provide clean water and energy from a self-sustaining source.

The possibilities for developing countries are the driving force behind this idea, but this technology is also potentially very advantageous for industrialized nations. We have already mentioned the advantages of this technology when compared to traditional methods. In addition to those characteristics which were already mentioned, E. coli has the added particularity of treating nitrates and heavy metals which are more particular to industrial nations. Due to the vast quantities of wastewater in industrials lands, one can also imagine that water treatment would create a hydrogen surplus which could be used for industry.

Millenium goals

The 189 members of the UN have set a series of objectives to be reached by 2015:

 *Reducing extreme poverty and hunger.*

 *Ensuring primary school level education for all.*

 *Promoting sexual equality and the autonomy of woman.*

 *Reducing child mortality rates.*

 *Tackling the health conditions for childbirth.*

 *Tackling HIV, AIDS, and a series of other illnesses.*

 *Promoting sustainable development.*

 *Putting into place a world-wide partnership for promoting development.*

These 8 objectives are specified in quantitative terms: for example

For almost a century, petrol has dominated the energy sector and hence many industrial sectors, notably transport. Developing alternatives is difficult in the face of the petroleum monopoly, especially when it comes to classical methods of producing hydrogen which are not as cost-effective as fossil fuels. However, synthetic biology gives us another way to produce hydrogen, but, unlike other ways, it is actually potentially competitive enough to make serious headway on the energy market.

The IGEM competition may fulfill a valuable role in encouraging the development of alternatives to traditional methods which always involve a trade-off between financial costs and environmental costs. Could it be that with synthetic biology we could have our cake and eat it too?

If we put aside ethical and ecological concerns for the moment, we can see that industry and other profit seeking activities can also be conquered by this technology (for further development, see chapter 4). All the concerned parties could continue to pursue their selfish interests without doing damage to each other. This could lead to the deconstruction of the traditional polarization of industry and ecology, at least where energetic resources are concerned. If we were to philosophically deconstruct these opposed poles, developing this alternative could even reduce the traditional gulf between the corporate world's interests and philanthropic interests.

It must be said though that the ideas developed here are not meant to be normative. We are fully aware of the dangers of idealism, and mentioning all the possibilities of this technology is merely a way to underline its potential power. Moreover, the mention of this deconstruction of the polar opposition is pure speculation and aims at highlighting the latent strength of the concept of a society governed by the production of clean hydrogen. In practice, this kind of social revolution is not likely and could even lead to concerns about utopianism. If positive change is possible, it will come about little by little, taking the form of a trend rather than a complete discontinuity. Indeed, sudden and extreme social change is usually best avoided.

As we have seen, the ethical issues are numerous. Philosophically, there is also much to be said. In fact, if this technology were to be rendered functional, it could change the position of the human subject in his environment. Currently, when it comes to ecological concerns, the traditional distinction between Nature (and its flora and fauna) and Man is as follows: Nature, as opposed to Man, produces no waste; everything changes but nothing is lost. Nature is a delicately balanced system which is constantly recycled and renewed. Philosophically, Man can be seen as the element which breaks nature's cycle, disturbing the system. This rupture with his environment is one of Man's essential characteristics and can be linked to a self-consciousness which is constructed in opposition to what is outside of the self.

The awareness of being an element in the environment is a fundamental difference between Man and plants and animals. Plants and animals can be seen as one with their environment, the essential building blocks that make up Nature. Animals live in an ecosystem to which they are indebted. They live in the ecosystem, but are also a part of the ecosystem, as essential to its make up as trees to that of a forest. In contrast, Man does not pay tribute to a specific ecosystem; he does not even live in an ecosystem, in the strictest sense of the word. An ecosystem is defined as follows: "the association of a community of living species (biocenos) and a physical environment (biotope) in constant interaction with each other"[14]. Man has managed to adapt to almost every type of weather and geographic conditions. However, even if not indebted to a specific environment, Man, as an inhabitant of planet Earth, depends on the environment, taken as a whole.

Nature can be compared to a huge perequation in which all the elements are constantly adjusting to maintain equilibrium. In this metaphor, Man is the unknown quantity which disturbs the equilibrium. The fact that Man produces waste is the crucial issue here. The idea of waste itself is entirely anthropocentric, as made up of artificial or artifactual and thus non-natural objects. Generally, waste refers to everything that nature can not break down and recycle in order to achieve its all important equilibrium. The vast majority of these elements are byproducts of Man's activities.

If we were to push our team's project to its logical conclusion, we would witness a new paradigm for the conceptual place Man can occupy in Nature. In fact, the almost complete treatment of waste present in used water is used here to produce energy directly, reproducing the kind of self-sustaining cycle found in nature. Through the decrease of the direct exploitation of natural resources and of the quantity of waste, Man can minimize the disequilibrium that he creates in the system. To go back to our comparison, Man could then create a new equilibrium in which he plays a part comparable to that of other species. He would then tend towards the equilibrium of a new system on an individual scale, rather than throwing the entire global system out of order, and what is a system at rest but the sum of its sub-systems which are all at rest? To return to our metaphor, we can now say that Man could reach the optimal equilibrium in his own system, contributing to maintaining, and not destroying, the natural system which is an extremely complex equation.

From this point, we can go on to a more heuristic philosophical development. If we accept the conclusions of the previous paragraph, their counterfactual is also true, implying that the disturbance of each sub-system can disturb the system, in this case, Nature, as a whole. This somewhat basic and obvious axiom makes it possible to develop a prospective reflection based on a fear heuristic[15]. What this means is projecting ourselves into a future,

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[14] « Ecosystème » *in Le Petit Larousse illustré*, Paris, Larousse, 2003, p.361, translated by the author

[15] JONAS, H., *Le principe responsabilité une éthique pour la civilisation technologique*, Paris, Flammarion,

more or less distant, by imagining the various consequences that may arise from the current situation. To a certain extent, this method is commonly used, notably in climate studies. The real philosophical interest here however lies in the interpretation of the results from an extra-scientific point of view. Basically, this consists in taking into consideration the results of our thought experiment from a human standpoint and not merely from the Cartesian standpoint. This method can be seen as a sort of philosophical modelization.

If we imagine the future and the result is fear or anxiety, the future we are headed for must be contrary to the aspirations of humanity. This is true because fear has proven to be the most efficient tool when it comes to avoiding the pitfalls of utopianism and hubris. If we take the current parameters of resource exploitation and the resulting pollution and interpret them, from a purely scientific point of view, to imagine the coming century, what we have may be sustainable for the human race but instills fear in our hearts. While we may manage to survive in a world where the air is compromised, the weather thrown out of whack and the majority of animal and vegetable species destroyed, the picture this paints is met with horror.

This alone is a sufficient reason to make an effort to escape the terrible inertia which pulls us closer and closer to the center of the ecological black hole, to the point of no return. Patching up the wooden leg that we are currently standing on will not help us; our inertia must be reversed as soon as possible. Despite this urgency, the current trend is to follow an ostrich policy, as evidenced by the continued massive exploitation of non-renewable fossil fuels, the use of which is already compromised itself and may soon compromise our very existence.

While it may seem that we have gotten off track, we believe that IGEM and our project specifically are directly linked to the energy and environmental issue, as they offer alternatives which are infinitely more advantageous than what is currently on the table. The beginnings of this project which concerns waste and hydrogen are a wonderful way to address, and perhaps even resolve, two of the major problems of the 21st century: the energy crisis and the intimately linked need to reduce pollution. By encouraging research in this area through the development of our prototype we hope to reconcile these two staggeringly huge challenges and solve them together.

2008.

VI.3 BIO-SAFETY, BIO-SECURITY AND SYNTHETIC BIOLOGY

Besides the ethical analysis that any genetic modification project demands for deontological reasons, the IGEM competition requires that we take the ethics of synthetic biology into account on a more formal level through the analysis of bio-safety and bio-security.

Bio-security is an issue which stems directly from bioethics. It refers to a series of established procedures designed to avoid the sanitary problems that can arise from synthetic biology and that are called into play by ethical concerns. Bio-security gives us the legal framework which is necessary to preserve the environment and public health in the face of the possibility of misuse.

Legally speaking, bio-security refers only to genetic engineering involving genetically modified organisms or pathogenetic organisms. In Belgium, bio-security regulation falls under the heading of work environment safety but bio-security and bio-safety standards vary from country to country. There are however some which are enforced on a European level or internationally.

In practice, bio-regulation is enforced by the prevention council and the bio-security head. The law requires that every laboratory where genetic modification or the use of pathogenetic organisms take place must appoint people to fill the two aforementioned positions. The legislation also requires the existence of a local bio-security committee whose job is to deal with the daily problems faced by the relevant institutions. Belgian law doesn't contain specific requirements concerning the composition of these committees, but they are generally made up of professionals who work in the field.

First thing, all laboratories must obtain an environment license, delivered by the competent regional authorities, before they can begin operations. The authorization is delivered after appraisal by the Bio-security and Bio-technology Service, a federal body. As an example of the concepts and instruments relevant to bio-safety we will now discuss the procedure that a lab must follow in Belgium.

Firstly, any installation where a confined activity involving micro-organisms or dangerous or modified organisms takes place must register with the competent regional authority. According to the installation and the level of risk of the activity, the business may need written authorization. Moreover, a legal decree stipulates that any request for an environmental license must include a risk evaluation of the research to be conducted. The SBB then plays the role of technical expert and expresses an opinion on the risk evaluation.

The SBB report must be given in with the licensing request. The file containing all the relevant information for a notification of confined use constitutes the bio-security report.

In order to make the information and authorization procedures easier and to reduce the amount of paperwork, the SBB has come up with notification forms and a user's guide. These are based on the relevant legislation but also on field experience in applying the laws.

Before beginning a new research project, the researchers must complete a detailed description of the project and its goals. Their description is then the object of a risk evaluation. The level of risk is measured on a scale going from 1 to 4. This risk estimate is based on the type of organism used (inoffensive bacteria, animal, virus, etc) and on the goals that the research is aiming for. The risk evaluation aims at identifying risks intrinsic to the activity but also the possible consequences for Man, for flora and fauna or for the environment generally speaking (for example, when using bacteria which are essential to biodiversity or in preserving topology).

The protection of the environment has its own enforcers, the environmental inspection agency, which is responsible for checking licenses and authorizations: the inspectors are authorized to seal a laboratory if it has been found guilty of illegal research. Refusing to respect the sealing order can lead to punishment and even to imprisonment of the guilty party. Belgian bio-security and safety legislation was reviewed in 2002, as was the legislation concerning environmental licensing.

In addition to the regulation concerning laboratory research, the development of genetically engineered organisms for industrial use requires a specific authorization process. Specific emergency and intervention measures have been established for genetically modified micro-organisms. These procedures can be implemented quickly thanks to the information contained in the forms that the researchers must fill out before beginning activities. This information is in fact crucial in case of emergency and includes such essential points as the type of organisms being modified, the safety measures of the lab, quantity, etc.

The policies we have listed are largely applications of European Union directives. EU directives are mainly established to avoid one country having to suffer the consequences of a neighboring country's activities, for example in the case of contamination through the exchange of goods. A number of international treaties have also been put into place, along with the mutual control needed to ensure that they are respected. This type of regulation is necessary if these treaties are to be respected, and their enforcement is in everyone's interest, as bacteriological contamination, once started, is virtually impossible to control. The relevant legislation on an international level is contained in the "Cartagena Protocol".

This protocol was ratified by the UN in 2000 and has been active since 2003. It aims at avoiding biotechnological risks and at gathering the data concerning these risks on an

international level thanks to an information exchange center. This center encourages transparency and accessibility of information, allowing for example for a healthier and more efficient management of commercial activity in developing countries.

The Cartagena protocol demands respect of the precautionary and prevention principles in the absence of scientific certainties in order to limit the risk. This is an understandable precaution because these risks, in certain cases, as in the development of GMOs for industrial use, could have repercussions that resonate on a national scale or beyond. To put it another way, French law puts it as follows: “the absence of certitude, given a specific state of scientific and technical knowledge, should not slow down the implementation of effective and appropriate measures which aim at avoiding serious and irreversible damage for the environment at a reasonable cost”.

The precautionary principle entered into the legislation in 1992. It is not merely a philosophical concept, but a normative imperative. Let us add that legally no one is authorized to manipulate organisms for pathogenetic goals, not even defense forces. Bacteriological weaponry is thus an area of research which is theoretically forbidden by law.

Bio-security and bio-safety norms include, beyond the legal aspects, a series of more technical directives aimed at the scientists who conduct laboratory research. These directives are the practical applications of the federal law on a more concrete level. They are mainly concerned with the management of biological decontamination. Biological decontamination involves treating air through the use of specific filters, treating liquid which may contain biocide solutions or detergents, the treatment of solids which must be incinerated and the treatment of elements which may be radioactive.

Despite these measures, zero risk situations do not exist. This is one of the reasons why it is so all important to evaluate and compute the risks. The precautionary principle is always meant to be applied in the field of biotechnology because the technicians are working with organisms which are often invisible to the naked eye and which can be dangerous because of their instability.

The bio-safety manual and the waste elimination manual are two deontological guides which include the legal imperatives to be respected when conducting research. They are not included in interior law but are necessary for the correct application of the directives in a specific institution.

This concise section on bio-safety and security is meant to be a short guided tour of the different practices in use. The extent of the measures concerned shows us to what extent biotechnologies are supervised. The most important point is to ensure that bio-safety is not taken lightly because inadequate risk management could lead to a catastrophe the likes of which we’ve never seen. It remains true that synthetic biology has much to offer us but in order to ensure that it does not become a poisoned chalice, it is important to be

careful. Norms and a surveillance and peer-review principle are essential tools in avoiding the misuse of science. The risk of instrumentalization is particularly present in the case of synthetic biology because this is a relatively young field. This means it remains obscure for many but is ever more ubiquitous. Bio-safety and bio-security regulations are absolutely necessary if we are to avoid, inasmuch as possible, scenarios in which a sorcerer's apprentice or mad scientist would have the ability to create a diabolical alchemy involving the worst poisons and most ravaging plagues.

VI.4 ISSUES OF INTELLECTUAL PROPERTY RIGHTS

The following chapter examines the question of intellectual and industrial property. This is one of the core issues of the IGEM competition. This pragmatic aspect of science and technology is relevant for every person taking part in the contest. As we've already mentioned, synthetic biology is a young field which is expanding at an incredible rate, making property rights a major issue.

Firstly, what exactly does the term "intellectual property" refer to? Intellectual property is anything that the human mind creates which takes a material form. It includes artistic creations in general and author's rights, on the one hand, and on the other hand, industrial property. Patents, brands, logos, models, and controlled designation all are intellectual property. Anything that can be patented will be referred to as an "invention" in the epistemological analysis that follows. An invention, as opposed to a discovery, can be patented. Discoveries concern pre-existing phenomena whereas inventions are the product of an innovative intellectual process. Among others things, products, processes (for example biological processes) and information technologies can be patented, whereas recipes and mathematical proofs cannot.

IGEM is mainly concerned with directly applied research, but theoretical research can also lead to results with potential commercial value. In any case, IGEM brings up certain questions surrounding patents. Patents are a very interesting epistemological subject, both from a practical and a philosophical point of view. They are what links theoretical science to applied science. valorization, both industrial and commercial, is in fact one of the primary goals of universities, along with research and education. The valorization of research often contributes to the societal good. This valorization is not a secondary goal, it is present as a driving force from the start, and is evident in the call to tender and the search for sponsors. This issue is present from the first results, making protection of these results imperative until they can be exploited.

Depending on the expected economic end result of a project, the funds which finance it will come from different bodies. Obviously, private firms will be more likely to finance research which leads to commercial applications. Intrinsic research conditions are thus not

always sufficient for it to be carried out. Very often, the successful accomplishment of the research depends on exterior conditions.

Three criteria must be fulfilled if a result is to be patented: firstly, the invention must be innovative. Secondly, the invention must have a technical effect which is non evident or resolve an old problem which remained unsolved. This is known as “inventive activity”.

Lastly, the point of an invention patent is to make the invention exploitable industrially. For example, a new surgical technique does not fulfill this last criterion because its application is not industrial.

The results of research conducted at a university are the property of that university. However, the researchers are named as inventors and have the right to a third of the financial returns. To be considered an inventor, one must furnish proof that one’s contribution played a crucial role in the development of the patented product. Students and technicians alike can be inventors. The university may have the property rights on all results, but this goes hand in hand with being responsible for all fees accruing from the patenting process.

Declaring an invention is a lengthy and costly process. A patent has extremely important advantages though. Firstly, it protects the inventors’ rights from unscrupulous competition. This is one of patent law’s raison d’être, because it encourages economic growth by providing an incentive to innovate. An exploitation monopoly is rarely the goal of the patenting process though, mostly, firms want to be compensated in the form of monetary permits.[16]Patents also encourage partnerships between the public and the private sectors. The first, universities generally, get financing from these partnerships, the second, businesses, get access to innovations. Patenting records also serve as a performance indicator and are used as criteria for allocating research contracts. Patenting also ensures financial returns from an innovation and makes the creation of spin-offs possible.

Obtaining a patent involves a complicated process which must be followed closely. If the innovation has been divulged, be it orally or in writing, to anyone before patenting, obtaining a patent is extremely difficult. Divulgation refers to any description detailed enough to make it possible for a member of the profession to recreate the invention. Depending on the field, divulgation can consist of a single sentence or several pages of complex instructions.

IGEM promotes open source knowledge, relying on intellectual honesty. However, certain unscrupulous businesses actually hire spies whose job is tracking down any unwary information leak which can make patenting difficult for the innovator. This involves tracking the internet, posters, articles, thesis, papers but also calls to tender and contests. This means

[16] Exlusivity licences, less common, are also a possiblity.

that any team who presents their project at IGEM risks compromising the exclusivity of their research if they haven't done the necessary paperwork beforehand.

Orally, divulgation can happen during a speech, a congress or even a professional conference if a confidentiality clause has not been signed. A tour of a laboratory or mailing of equipment can also be ways of revealing too much. Divulgation removes information from ownership and puts it in the public domain. Anyone is then free to use it and share it. Secrecy until the patent is applied for is thus the best way to avoid this problem. Ideally, any information published prior to the research should contain no more information than what is in the patent application. Once a patent is applied for, there is an 18 month delay before the invention can be safely fully divulged.

Declaring an invention involves several steps. A form describing the invention must be filled out in order to determine if the innovation is covered by a previous patent. This is made possible by the existence of databases. Once this is ensured, the inventors are named. this process relies on the laboratory manual. An invention patent is characterised by the following caracteristics :

- It is an intellectual property title, giving the inventors the right to authorize or not the exploitation of the invention by another party. The right conferred is not a right to exploit, but a right to stop others from exploiting.

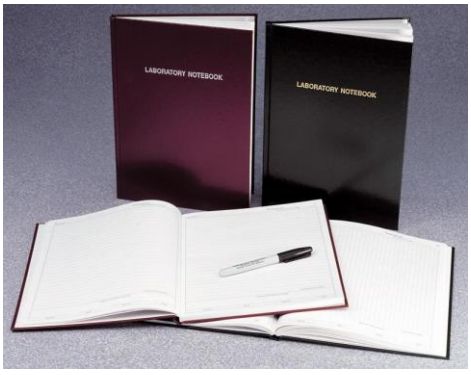
- It is limited geographically. The inventor determines which national markets must be covered by the patent. For thirty months prior to the choice of countries, the patent covers the entire world.

- It is limited in time. 20 years is the standard period for all inventions except drugs which have five extra years to make up for the time lost on trials.

- It is non-renewable; once its time has run out, the invention automatically becomes a part of the public domain.

The laboratory journal

This is a reference book which allows researchers to date and record their research. It must mention the name of the users and of its owners and can serve as proof when invention paternity is contested. It is an extremely valuable tool for the valorization of research on every level. This journal also plays an important internal role, making coordination and development easier.



Patents are usually applied for with the aim of getting monetary returns from inventions. There are different ways in which patenting helps to achieve this goal. Through licensing agreements, the invention can be exploited by a second party who "rents" the property rights. Patents can also be sold, or spin-offs created. Spin-offs are new firms which can exploit the patent but which depend on the mother firm. New research contracts are also more likely if successful patents have been applied for in the past. Monetary compensation can take three forms: upfront payments, royalties (a percentage of the profits), or milestones, which are fixed amounts to be paid when certain benchmarks are hit. The inventor can thus ensure that his or her licence will benefit him or her even in the case where the other party doesn't manage industrial exploitation of the product.

To sum up, patenting is a difficult process and as such, may seem like a burden to engineers. Furthermore, the fact that the driving force of research is pecuniary means that research without industrial applications can sometimes be left by the wayside. However, without the legal protection of intellectual property rights, there would be no incentive to innovate and hence no demand for engineers.

VI.5 A FEW WORDS OF CAUTION FOR ENGINEERS: THE DIFFICULTIES OF MARKETING THE PRODUCTS OF BIOTECHNOLOGY

"There is nothing more difficult to plan, more doubtful of success, nor more dangerous to manage than the creation of a new order of things… whenever his enemies have the ability to attack the innovator they do so with the passion of partisans…"

Niccolo Machiavelli, *The Prince*[17]

The purpose of this chapter is not to provide a complete marketing plan for *hydrocoli*. This type of market study requires quantitative data that our team cannot fund. The purpose here is to show the difficulties of marketing even a seemingly sure thing and to give entrepreneurs food for thought. On today's "small is beautiful" business horizon, many R & D professionals attempt to save money by bringing their products directly to the market, without consulting management and marketing professionals. This is not inherently dangerous, but it is always useful to stop and think before attempting to patent and sell a good. In this paper, we review a series of articles and case studies which we hope will provide an overview of the main issues of marketing biotechnologies. we have focused on those which relate to our team's project, in the hope that bio-engineers will come away with

[17] MACHIAVELLI N., *The Prince*, Penguin Books, Baltimore, 1961.

a sense of the complexity of consumer attitudes and the difficulties of getting consumers to accept an innovation.

5.A SOME USEFUL LESSONS FROM CONSUMER ATTITUDES TOWARDS GENETICALLY MODIFIED FOODS

The following analysis of likely consumer attitudes towards the end result of the ULB project is largely based on consumer attitudes towards genetically modified foods. The reason for this is that a lot more research has been done in the area of food production than in other areas where synthetic biology is applied.

The focus on foods in the literature can be explained by the fact that what consumers eat affects them directly, and they are therefore likely to have stronger, and easier to measure, opinions on this subject than on more obscure applications of biotechnology. Extending the research on GM foods to *hydrocoli* seems reasonable however when you consider that a fair share of consumer dissatisfaction with GM foods stems not from the experience with the product as such, which is in fact likely to be on average more satisfactory than experience with non-GM foods, but from concerns about genetic modification and biotechnologies generally.

These concerns range from a vague uneasiness with the idea of genetic manipulation to full-blown terror of the consequences for the environment and for our health. Consumer attitudes towards gene technologies, even if originally studied in the context of foodstuffs, can clearly be extended to include consumer attitudes towards *hydrocoli.* Furthermore, as a technology that could eventually be directly applied by individual heads of households, it could be argued that *hydrocoli* fits into the category of consumer goods and services.

Concerns about *hydrocoli* are likely to stress risks to bio-diversity and the environment rather than health risks, but the risk aversion analysis remains relevant. Having said this, perceptions of what is unhealthy to ingest are not straightforward but depend on complex cultural conditions, which means there may be consumer concern over drinking water which was purified using genetic technology. These specific concerns are therefore directly relevant to the research on GM foods.

While many activist environmental groups are lobbying against genetically modified foods, most members of the general public do not seem to have strong opinions on the subject (Scholderer and Frewer, 2003). This is true especially in the US, where GMO's are mostly regarded neutrally or favorably. In Europe, public opinion tends to be more firmly against GMOs, which explains the partial moratorium on their cultivation and sale

5.B THE EUROPEAN VIEW

Although the use of transgenic crops was approved in the EU in 1995, following the "mad cow" outbreak in the late nineties, food origin became a major public concern. This resulted in a halt on approving new GMOs for import and cultivation in 1998. The ban was partially lifted in 2004 after protest from the US, who claimed the ban was a protectionist measure, violating trade agreements between the two powers. Despite these protests, the EU is still moving slowly and reluctantly to approve the cultivation of transgenic crops. European pressure groups, made up mainly of bio-tech firms and farmers, are now lobbying for an easing of anti-GMO measures.

Things are moving slowly, but they are moving. For now, even if only one kind of transgenic seed is currently legal for cultivation in the EU, the farmer's unions have been adding steadily to the list of permitted transgenic imports, despite powerful lobbying from NGOs such as Greenpeace. European farmers argue that the partial ban on GM crops makes it impossible for them to attain sufficient yields to keep their prices competitive.

5.C THE US VIEW

US consumers look upon GMOs much more favorably than their European counterparts, making the US the leading pioneer in terms of novel food technologies. Before the public outcry at the beginning of the millennium, biotechnology was seen by many as a panacea which would end food and energy shortages as well as pollution of all stripes. Executive Order 13134, signed by President Bill Clinton in 1999, stipulated the goal of tripling the US's use of bio-based products by 2010 through an increased use of biotechnologies. Indeed, since 1990, bioengineered products have been a part of the consumer landscape.

The FDA is currently looking into the approval of the first genetically modified animal to be marketed: the salmon pioneered by AquaBounty, referred to by critics as "frankenfish", is destined to appear on supermarket shelves in as little as two years. All is not smooth sailing however on the biotech horizon, as there remains significant difficulty regarding the commercialization of "frankenfoods", due to lobbying from environmental groups and to consumer unease.

Such environmental activism is not difficult to understand, as the true risks of biotechnologies for biodiversity are not currently known. Consumer unease leaves some baffled, but it is generally considered that it stems from a lack of information, which leads biotech firms to emphasize the information-deficit aspect in their advertising. Since less than 2% of the US population is currently involved in the agricultural production process, it

stands to reason that a large portion of the general public feels left out of the food production realm and perceives itself as insufficiently informed. Indeed, many aspects of biotechnology remain mysterious to the general public. Many laymen even believe that, following widely mediatised issues such as the monarch butterfly controversy[18] biologically engineered foods are not sold commercially to the general public. This is clearly not the case, as it is estimated that up to 70% of processed foods sold to American consumers contain at least one ingredient produced through biotechnology. In fact, corn and soybean crops rely heavily on bioengineered strains which are more pest resistant. In addition, cheese production relies on an engineered enzyme and bioengineered yeast is widely used in breads. The fact is that misinformation or information gaps tend to drive perceptions and slow the potential payoffs from R&D in bioengineering.

5.D RECOGNIZING INFORMATION ASYMMETRY IN BIOENGINEERING AS A DRIVER OF ATTITUDES

The science behind bioengineering is complex, but questions of regulation, labeling and the risks inherent to synthetic biology are no less hazy for the average consumer than the organic chemistry behind the product. There is clearly an informational asymmetry[19] here which may indeed explain in part the public's resistance to the notion of bioengineering. One could, and many do, argue that there has been insufficient effort made to educate consumers about bioengineering, when a sincere discussion of the risks and benefits of such technologies would perhaps have led to a more favorable image.

Some studies support the idea that fear of biotechnology is a direct consequence of an information asymmetry (see Traill *et al.*, 2004; Baker *et al.*, 2001). Traill used experimental auctions in the US, England and France, to show the effect of information shocks on willingness-to-accept[20] money in exchange for consuming GM foods. Subjects were recruited and given two cookies, one clearly labeled as containing GM foods. Researchers then measured, through bidding, how much money the subjects demanded in exchange for having to eat the GM cookie. The subjects were then randomly assigned to one of four treatments: information focusing on the environmental benefits of biotechnologies, information concerning health benefits, information concerning a world benefit in the form

[18] see for example *the Entomological Society of America Position paper on transgenic insect-resistant crops: potential benefits and hazards*

[19] The term " information asymmetry" refers to a situation wherein one party of the transaction has more information than the other party.

[20] Willingness to accept is a commonly used econometric tool, used for measuring attitudes; the attitude holders are asked how much they would pay, or in this case how much one would have to pay them, for a non quantitative item.

of drought resistant crops or no information at all. Following these treatments, their willingness-to-accept was measured again. Traill found that information about world, health and environmental benefits significantly decreased the monetary compensation that subjects demanded to eat the GM cookie. The primary conclusion of this study was that information concerning the benefits of GM foods makes consumers more willing to consume them. This is perhaps not surprising; it gels with the common sense view that if consumers do not want to eat GM foods it is because they just don't know how good they really are. However, this study also found that the influence of attitudes adopted by the subjects before the study began had a significant influence on their reaction when faced with information treatments.

Another study supporting this standard view is Boy, 2001. Boy surveyed the attitudes of European consumers by using the *Eurobarometer* results to establish the relative importance in attitude formation of the perceptions of utility[21] and risk. He found that, no matter how great or small the risk perceived, consumers will not even consider a product if a minimum of utility has not been shown. The econometrics used by Boy clearly showed that the perception of utility is more important than the perception of risk in forming attitudes towards products which result from biotechnologies.

This view explains the lack of consumer enthusiasm for first-generation biotech goods entirely with the lack of directly perceivable benefits for consumers, making a case for the usefulness of the traditional biotech marketing strategy which aims at informing consumers of all the benefits in store for them (Marris, 2001). This position can be linked to the "deficit model" of public understanding of science, which basically assumes that any failure of consumer attitudes to align with those of R & D professionals can be blamed on an insufficient effort to "educate" an ignorant public.

5.E DEALING WITH INFORMATION ASYMMETRIES

There are many tools available to those who wish to close an information gap. Assuming that information is the way to consumers' 'hearts', choosing the public-consultation mechanism is no simple task for the policy maker. Special committees are often established to make these decisions. These are usually made up of technicians, scientists, ethical experts and laymen. Their main roles are testing public opinion, informing the public if requested to do so and consulting the public when necessary. There are different mechanisms one can use to accomplish these tasks. For an analysis of these mechanisms and their respective advantages, see Leroux *et al*, 1998.

[21] Utility is the term economists use to denote the satisfaction one gets from a particular item.

Putting aside the question of how to consult and inform the public effectively, research from sociology and consumer science shows that the solution to consumer mistrust may not be as simple as designing communication mechanisms that will reach a maximum of consumers. The literature shows that attitude formation is a highly complex process. Scholderer and Frewer (2003) reached the highly counter-intuitive conclusion that consumers will actually be less likely to choose GM foods after an information shock, *regardless of whether the information highlights the risks of GM foods, their benefits or both in equal measure.* This communication paradox highlights the important difference between product choice and attitudes.

Scholderer and Frewer found that information brought about no significant change in attitude, be it positive or negative, whereas it did affect willingness to choose GM products. This willingness turned out to be negatively correlated to information received, regardless of what kind. This can be explained by an "attitude activation process". This means that information, negative or positive, activates attitudes that were already held prior to the confrontation. Moreover, those who hold positive attitudes are found to be more likely to accept new information than those who hold strong negative attitudes. Putting aside the econometrics involved, this can be explained by a cursory examination of human nature. It seems obvious that adoption of a negative attitude can be both a consequence and a cause of mistrust of the authorities involved. The findings of this team seem to indicate that current biotech advertising strategies may in fact increase consumer aversion to buying bioengineered products, without having a significant influence on their attitudes.

5.F CONSUMER RIGHTS: A LOOK AT AGENCY

We do not mean to suggest that consumers should not demand and receive relative transparency concerning biotechnologies, but merely that constantly bombarding the public with reminders of the benefits of biotechnologies is perhaps not an efficient marketing strategy. Anyone who can potentially be affected by a new technology should be allowed to express concern, request information and be involved in the decision-making process. To deny this basic right is to deny the merits of democracy. The problem arises because this decision-making process is not clearly defined in time or in space. Our society is ruled by supply and demand. Whether one accepts this as beneficial to us all or sees it as a nail in our collective coffin, it is undeniable that we are seeing the end of big government, and markets are taking its place. Ultimately, the market will determine what can or cannot be produced and consumed. We do not mean to adopt a holistic approach; the market is made up of individual consumers. However, in terms of consumer concerns, the rule of supply and demand has the same basic flaw as democracy: namely, majority rule is not synonymous with representation. This fundamental issue makes it almost impossible for concerned consumers to take any real action in the face of global acceptance.

This is a serious quandary in the case of biotechnology because it may have the potential to change the world by providing resources that are so renewable as to be virtually inexhaustible. At this stage, many reason that we cannot afford to ignore this kind of opportunity. Others however fear biotechnology. Whether this fear is justified remains to be seen, but either way, in practice, it is no match for the imperative of change.

It is interesting to note that some more or less harmful technologies slip by potentially influential consumers without causing a stir, whereas others, such as biotechnology and nuclear energy, fall prey to negative buzz. While we in no way wish to denigrate the concerned consumer or her right to play a part in the debate, it is interesting to note that the appearance of, for example, the SUV raised no ruckus at first, despite risks of possibly the same magnitude, at least when applying the precautionary principle[22], as those of biotechnologies.

Standard dogma explains this with the hypothesis that agents are more likely to accept risk when it is perceived as being voluntarily taken. Empirically, the illusion of agency or lack thereof when it comes to risk taking has a huge impact on risk aversion. Wildavsky (1998) points to the example of a socially minded public health worker who will gladly work intimately with patients infected with HIV and AIDS, but expresses absolute refusal when it comes to GMOs in her food intake. The standard interpretation of this phenomenon is as follows: the public health worker accepts any risk inherent to her chosen activity because she feels responsible for those risks; she alone has weighed the pros and cons and made her decision. However, the attempted introduction of GMOs on the market without an *ex-ante* public discussion leads to the loss of this feeling of agency, leaving the consumer feeling as though a force stronger than her has been trying to "put one over" on her and consequently to an overestimation of the risk involved.

Wildavsky modifies this standard interpretation of risk aversion, pointing out that the line between a voluntary and an involuntary risk is very fine. He states that the way one perceives risk depends in fact on ones 'culture'[23] and that according to the adopted culture, the agent will define her risks *a posteriori* as voluntary or involuntary. This is interesting because in the standard interpretation of risk aversion, the consumer can be the object of an involuntary risk and the subject of a voluntarily taken risk. Once she notices the risks she did not sign up for, she will resist being subjected to them.

In Wildavsky's interpretation, the crucial difference is that the consumer exercises a subconscious agency in her determination of which risks are voluntary or not. For example, the public health worker in question is likely to be politically left-leaning; in Wildavsky's

[22] The precautionary principle is that which states, basically, better safe than sorry.

[23] Culture is used here in the specific wildavskian sense. Wildavsky highlights four main cultures, which can be assimilated to political beliefs.

terms, she believes in a non-hierarchical culture. She perceives big business as being more powerful than she is and this in turn bothers her. The products of this business will be subjected to closer scrutiny than for example someone she perceives as needing her help. In this scenario, her non-hierarchical belief system will lead her to take greater risks and she may gladly stand between them and a bullet.

Our purpose here is to show that the issue of consumer mistrust is far from being a simple one and is not easily solved; the image of a product, of the business which produced it, the area in which it is produced and the market it is aimed at are all factors that can have a significant influence on the levels of transparency and accountability that the public will expect, demand, and for which they will lobby.

5.G LABELLING GOODS TO REDUCE AVERSION

A lot of legislation exists to protect the consumer's right to relative transparency, but consumers are seldom fully informed. Despite this, a vague awareness of the existence of labeling restrictions may give them the illusion of agency. We can read the word "organic" on a label, and then decide to put that object into our cart or to opt for a cheaper brand. We then accept the health risks of non-organic food because we it was our decision to buy that product.

However labels refer to ingredients and nutritional content, and in certain cases origin, but not to method of production. This is a central issue in the case of biotech products, because studies have fa€iled to find any unwanted difference in the actual composition of the foodstuff, hence in the health risks, making labeling a moot point under current directives. In the EU however, any food containing more than 0.9% of GM ingredients must be labeled as such.

5.H TECHNOPHOBIA

In addition to perceived health risks, there is the issue of general technophobia. Bernett *et al* (2009) point to two prevailing myths surrounding technology. One of the two prevailing myths about technology is that is it can lead only to evil. The argument goes something like this: Man was born in a state of purity, close to nature, and was then brought increasingly further and further away from this innocent state by technology and science, leading to the imminent extinction of a fallen species. While this position does not rely on hard facts, it should not be dismissed or disrespected. Researchers and those responsible for the commercialization of the products of technological research should accept part of the responsibility for consumer mistrust. In fact, historically, the consumer has had no agency in the appearance of innovations on the market; her agency relies solely on her actions as a buyer.

Moreover, Bernett makes the important point that the other prevailing myth is just as false, just as dangerous and also to be avoided: that which states that technology is the panacea, the perfect way to work out the kinks of modernism. This position is exemplified by the current enthusiasm for "bridging the digital divide"[24].

Bernett argues that the hard data does not point to the introduction of technological innovations as necessarily bringing social benefits. In fact, case studies showing the opposite are not hard to find (see Dutton *et al.*, 2005 or Saint-Paul, 2008). In his seminal book, *Diffusion of Innovations*, Rogers (1995) uses several important case studies[25] to show that innovation can have devastating effects and makes a case for the need for a complete study of the circumstances of said innovation before implementation.

5.I INNOVATION, THE "INDUSTRIAL RELIGION"

We make this point not only in the interests of avoiding unexpected social costs, but also from a purely commercial point of view. Consumers resist innovation; in laymen's terms, people do not like change. This is the point made by the introductory citation and the quantitative data support Machiavelli in this. This phenomenon is known as inertia. Decisions and attitudes, once made, are hard to turn back, although certain influential agents can aid this process, which explains the focus on "buzz"[26] advertising and attempts at garnering the favor of "e-fluentials"[27]. Brand image is inert, and genetically modified products currently have a negative brand image, especially in the face of the growth of the market for "organic" or "natural" foods.

For any business, innovation can mean survival. Innovation has even been called the industrial religion[28]. In view of this, it seems that biotech entrepreneurs and businesses

[24] For details concerning the ubiquitous enthusiasm for bridging the digital divide, see AVGEROU C., CIBORRA C. and LAND F. (ed.), *The social study of information and communication technology*, OUP, Oxford, 2004.

[25] The introduction of snowmobiles in Lapland, for example, had a terrible effect on the local economy and on social ties. It effectively destroyed the careful balance of the local economy, which had relied on reindeer for both meat and transportation until the snowmobile was marketed.

[26] Buzz advertising is the attempt to influence potential consumers by having their peers voice good opinion of the good or service.

[27] E-fluentials are those who effectively control the buzz on the internet.

[28] Survey Innovation in Industry in *The Economist* , 17 March 1999.

should have no trouble sailing prosperously through the 21st century in a context where the old ways have proven unsustainable and the stage is set for new ways.

The validity of the innovation itself however, does not guarantee prosperity or even survival. Public resistance is hard to predict, and start-ups do not tend have the marketing expertise or resources needed to gather all the necessary data or to put this data to good use. Examples of seemingly sound investments which did not deliver as expected include the Concorde and the Dvorak typing keyboard[29]. Why wouldn't consumers want to travel twice as fast? Why not switch to a more efficient keyboard?

5.J WHY INNOVATIONS FAIL

Rogers' book opens with a case study which is particularly relevant to us, that concerning the attempted introduction of boiling water prior to drinking it in a village in Peru. The Peruvian government planned to significantly reduce the incidence of water-contamination related diseases in the country by sending social workers to isolated villages to teach the locals to boil their water before consumption. The plan seemed straightforward enough; the technology was simple, accessible, free and had clear benefits to the policy makers. However, the attempt at introducing water boiling failed. Two main issues, ignored by the planners, contributed to this failure.

Firstly, local customs and beliefs included a complex understanding of food temperature. Some foods were perceived as inherently hot, others as inherently cold. The idea of germs as explained by the social worker was not as convincing as these age-old customs. This mistrust of new information was aggravated by the second cause of failure: the insider-outsider phenomenon[30]. The social worker, as a native of a big town, was perceived as intrusive and her attempts at advising the locals as sheer arrogance. Indeed, this is perhaps understandable after a long history of a "daddy knows best" approach to humanitarian aid. The level of trust that people place in the innovator is perhaps the most important factor influencing adoption or refusal.

This case is directly relevant to *hydrocoli* for two reasons: firstly, because in this case, cleaner water was made available through the use of a straightforward technology, but this presumably fail-proof innovation still failed. Secondly, the importance of trust in the innovator has direct consequences in the case of big biotech businesses, whose profit-seeking nature causes many to suspect their motives at all times, even when they are involved in humanitarian work. It has been shown that French consumers actually mistrust

[29] The Dvorak keyboard was designed, unlike its alternatives qwerty and azerty, for maximum efficiency. The force of habit led it to fail however, and it is not used.

[30] The insider-outsider phenomenon refers to a situation in which people perceive some as being in the know and others as being outside.

companies who engage their social responsibility by doing charity work more than those whose stated goal never varies from pure profit-seeking (Hamilton *et al.*, 1990).

The importance of marketing an innovation properly can therefore not be underestimated, no matter how positive the consequences for the consumer may be. It is not enough to provide a more efficient, healthier, more sustainable or better quality good or service; consumers are not always fully aware of these qualities. Even if told, they may mistrust the teller.

The question of adoption or refusal of an innovation cannot be answered simply and it is not our purpose to explain the complex reasons for the refusal of adoption of seemingly sure things. We merely cite these examples as a warning to engineers: it takes more than venture capital and a great idea to be a successful entrepreneur. Common sense is not sufficient to explain or predict consumer actions and attitudes. We cannot stress the importance of a market study enough. A firm's viability in the market can depend solely on its ability to market its products efficiently. To take an example from another rapidly developing sector, IBM is a striking instance of the importance of marketing for survival; IBM's personal computer, in many ways inferior, in terms of performance, to substitutes on the market, survived, perhaps solely, thanks to brilliant commercialization and marketing strategy (Dodgson, 2000).

5.K IS SAVING THE ENVIRONMENT THE WAY TO CONSUMERS' HEARTS?

Marketing is not to be confused with advertising. Marketing includes all processes that aim at meeting consumer needs. Sometimes, a market study may find that there simply is no market for a developed good or service, leading to a discontinuation of the research. Standard marketing dogma points out the following steps: identifying consumer needs, developing a product which can fulfill those needs, then advertising the product (in this modelised world, advertising is reduced to informing the public of the product's existence); naturally, once consumers are aware of the existence of a way to make them more comfortable, they will buy the good or service until their needs are met, at which point the market is saturated and businesses will need to identify a new consumer need, etc. (Gauthy-Senechal and Vandercammen, 2010, or almost any marketing manual).

However, although Western consumers with spending power may currently be feeling the effects of food and energy inflation to a certain extent, they are still basically in a comfortable position. Biotechnology offers solutions to problems that we may be aware of, but that are not (yet!) pressing in our day to day lives. We may be conscious of global warming, but there is still a market for South African grapes and sports vehicles. Therefore, while the potential demand for environmentally friendly products should be huge, as we mentioned previously, these products still need careful marketing.

There is a serious asymmetry of information problem here, because to the consumer, while two pears might taste alike and two plastic bags may carry the same load, the environmental impact of one can be much lighter than that of the other. Businesses can thus not rely on the standard marketing dogma, which aims to identify what customers want and then to provide it. Indeed, it is safe to assume that people want to survive on this planet without serious sacrifices in terms of living standards. This may be achievable through biotechnology, but how to integrate this long term effect into the consumer's choice between two similar tasting pears? In fact, while it may be obvious that people prefer a product which is environmentally friendly, sustainable and healthy, these three product characteristics cannot actually be experienced through consumption of the product. Fazio *et al* (1989) highlighted three conditions which need to be fulfilled in order for this type of characteristic to influence consumer choice:

1) access to external cues which link the product to the quality (product labeling and advertising);
2) positive perception of the quality and activation of this perception when considering the product (this is extremely difficult in the case of biotechnologies because some studies show that GMO's may be perceived as "inherently harmful" by consumers (Scholderer et al., 1999));
3) the belief that there is in fact a link between the product and the quality in question.

These conditions, in short, refer to internalization, in the eyes of consumers, of the benefits of sustainability, environmentally friendliness and health into the good itself as found on the supermarket shelf. It is delicate, but if a good or service is to be marketed solely on the basis of its sustainability or health benefits, these criteria must be met.

5.L CONCLUSIONS

In conclusion, while gene technologies may offer a way out of the mess the human race is undoubtedly in, if they are to become commonplace, it is absolutely essential that engineers work closely with all factions of society and with professionals from various fields to undertake a complete analysis of the ramifications of their innovation. The ramifications which must be taken into account include those elucidated through economic, environmental or sociological analysis. This must be done not only to avoid harmful applications of the developed good, but also to avoid the real or perceived issues that are a source of concern to consumers. All these elements must be taken into consideration by the innovator, if only in the interests of seeing his or her good succeed in a market which is increasingly complex.

VII. ECONOMIC AND INVESTMENT OPPORTUNITIES OF *HYDROCOLI*

Despite the slow rate of change in both technology and consumer habits, it is now widely accepted that energy is one of the biggest issues faced by humanity today. Over the last fifty years, the world's population has more than doubled, and its energy consumption has followed suit. Moreover, the economic growth of the BRICs[31] means higher standards of living for billions more people. This increase in living standards goes hand in hand with an increase in the demand, and hence the production, of energy.

Since the industrial revolution, the production and storage of energy, as well as their byproducts, have been problematic, causing huge costs which prices rarely reflect. The damages wrought upon natural resources and human lives have consistently been left out of market mechanisms. This type of situation in which the price for consumers and the cost for producers fail to reflect the actual social cost is known as a negative externality. The textbook example is that of a steel producer who dumps sludge up the river from fishermen, affecting the fish population upon which the fishermen's livelihood depends. Externalities arise when markets fail to internalize the actual costs or benefits of a good, hence their name.

The main sources of energy throughout history have all presented negative externalities. Coal could be extracted cheaply but the release of greenhouse gases and the damage to the underpaid miners' lungs constitute important negative externalities. Sperm whale oil is another good example; again, whaling was an incredibly dangerous profession and the near-extinction of whales is a potential threat to biodiversity. The most striking example for our generation is, clearly, fossil fuels and global warming. Oil is still relatively cheap despite its actual costs being through the roof, in terms of the environmental debt we will leave our children and, albeit debatably, the wars being fought to insure that oil is available in plentiful quantities.

Governments are scrambling to find a solution to the energy crisis, but traditional policy tools fail in the face of the problem. The problem of climate change is global, leading to negotiation costs that are through-the-roof and to property rights debates that can never be satisfactorily resolved. Vaguely defined property rights make adequate regulation impossible, be it through price or quantity fixing,. Some theorists draw a parallel between

[31] Brazil Russia India China, four countries which are experiencing relatively recent and significant economic expansion.

the current environmental crisis and the Great Depression and express concern that a "green dictatorship" will arise (see Radcliff, 2002), much as communist dictatorships arose in the face of a keenly felt economic crisis. Barring that drastic outcome and assuming that consumers and businesses are not likely to spontaneously change their ways, no matter how serious the risks we face, it seems the most viable solution to the energy crisis is not less of the same but more of something different. As possibly the only way to reduce greenhouse gas emissions and land-use, the development of alternative sources of energy is a crucial issue for humanity. Government action may be able to influence our habits, but at too slow a rate, as governments are concerned first and foremost with the instant gratification of the electorate (Mankiw, 2008).

Besides the democratic state's inherent inability to take drastic action, generally speaking governmental power is dwindling throughout the developed world as the range of what is perceived to be the public arena is reduced through the privatization of services. This privatization trend has many worried, but several authors show that it can lead to important gains in efficiency (see Estache *et al*., 2001). We will discuss the case of Argentina where the privatization of water utilities has been shown to increase social gain by decreasing child mortality.

Neo-classical economics holds that negative externalities can be internalized in the market under certain conditions, and it is only when these conditions are not met that the State must step in and use tax and subsidy instruments to reach a social optimum. This standard dogma holds that there is a socially optimal level of pollution, different from zero, because the social cost in terms of jobs and other social benefits which arise from industry is at some point higher than the social gain from reducing pollution. However, in the presence of a competitive market for alternative energy sources, might not these issues resolve themselves? Private entrepreneurs are working on developing ways to produce clean energy in order to be competitive in what is possibly a very profitable emerging market. The competitive market may, in this case, manage to remove the reliance on altruism and long-term planning which the human race finds so difficult to survive on.

In 1970, while giving a talk at the General Motors Technical Center, John Bockris coined the term "hydrogen economy". He was referring to a future of moving away from fossil fuel dependency towards an economy in which the main energy source would be hydrogen. Forty years later, we still rely heavily on fossil fuels. Hydrogen does indeed present many advantages when compared with other energy sources but it is an energy carrier, not a primary energy source, so the energy in question must be generated through wind, solar, or fossil power and then stored in the hydrogen molecules. These technologies are thus a better, but not an ideal, alternative to petroleum-based energy. However, biotechnology may, in the not too distant future, provide a way to produce clean hydrogen from elements that are otherwise undervalued, in our case, wastewater. If we are to accept the fact that our fossil fuel economy is no longer sustainable, the potential demand for non

fossil energy sources is enormous. The development and implementation of *Hydrocoli* is thus potentially a very profitable investment.

Besides the undeniably sound future of the alternative energy source market, *Hydrocoli* presents other investment advantages. Access to clean water is an essential standard for survival, making the demand for clean water inelastic when incomes face a downward trend (Galiani *et al., 2003*). While some uses of water are among the first luxuries to go in the case of shortage (recreational services such as golf courses and swimming pools), there remains an incompressible level of water demand[32] for drinking and hygiene purposes. Moreover, the possibility of future scarcity of this most basic resource is increasingly obvious and the consequences of such scarcity would be drastic for every sector of the economy. This impending scarcity leads some to refer to water as "blue gold" (Barlow and Clarke, 2002) meaning of course that those who retain the property rights to clean water would effectively be the oil magnates of the future.

Because of the link between water and health, the market for clean water generates positive externalities for all concerned. Many water washed diseases as well as diseases carried in polluted water can be eradicated through access to clean water, reducing health costs and making for a healthier and larger labor market. Moreover, inefficient treatment of waste can lead to the pollution of other resources when it is released into the environment. Stagnant pools of waste are also breeding grounds for various disease carrying insects, and the treatment of the waste can thus reduce the risk of such diseases, particularly malaria.

Traditionally, another characteristic of the water utility market is the huge fixed costs involved. The infrastructure needed for water treatment, evacuation and distribution is considerable and costly, under current technological conditions. It is estimated that, in 1994, fixed costs represented over 80% of water service costs in the UK (Armstrong *et al*, 1994).

These characteristics of the water market mean that traditionally, water utilities have been considered part of the State's responsibility, and have been financed and regulated by the authorities. This system is generally perceived as being more efficient in terms of social costs and gains. However, the increasing deregulation over the last thirty years has led certain countries to privatize as many sectors as possible. There remains a lot of mistrust in the face of this deregulation, as privatization is, to many people, synonymous of higher prices, lower quality services and more inequality. As a utility which demands the presence of large infrastructures, and in the presence of such important positive health externalities, water treatment may seem like the perfect example of a service which should be regulated by the state. Studies show however (Estache *et al*, 2001) that privatization, under certain circumstances, can lead to significant gains in revenue and well being.

[32] The phrase incompressible demand refers to a basic level of demand. Survival goods have an incompressible demand because they are the very first things a household will spend their resources on.

Galiani *et al* studied the impact of privatization on water network connection rates and child mortality rates in Argentina in the 1990's. This study showed a negative relationship between child mortality rates and the privatization of water utilities. Galiani controlled for unbiasedness, robustness and statistical significance of his conclusion, and found that this negative relation holds, with decreases of child mortality rates varying between 4% and 10% for an increase in privatization, depending on the econometrics involved.

These results may surprise some, because as mentioned above, privatization is expected to lead to less equity, not more. It is clear that these results cannot be generalized without caution as the basis of a case for privatizing water treatment and provision. However, they do highlight the important fact that the assumption of social gain obtained from the intervention of the State in markets relies on the assumption of a relatively functional, transparent, efficient and well-meaning State. Barring this, privatization can logically be the most beneficial, cost-effective and egalitarian solution.

Clearly, in the case of a monopoly or oligarchy, the consequences of deregulation can be absolutely disastrous. The word disastrous is not chosen lightly, as, in the case of Argentina, among many others, there is a direct impact of water provision on child mortality rates. These potential ramifications of insufficient competition on the water market partially explain the reluctance to privatize it. Monopoly and oligarchy situations are more likely when the fixed costs of the activity are high and when the infrastructure needed is considerable, because high fixed costs make exiting and entering the market more difficult, leading to static prices. However, to the extent that *hydrocoli* needs no specific infrastructure, and as the costs are exclusively linked to research and development, this technology makes privatization an easier alternative and guarantees competitive prices, assuming of course that the relevant intellectual property is rendered open-source. These characteristics of *hydrocoli* mean that when reliance on the State fails, water treatment can be privatized efficiently, cheaply, and quickly.

The characteristics that make *hydrocoli* easy to privatize also make it possible to create self-sufficient agricultural systems in rural areas. Farmers could set up a *hydrocoli* facility, thus having access to clean water and organic fertilizer with little or no transportation costs. Quasi abandoned areas could thus return to their former state of agricultural and economic activity.

Rural exodus and brain drain are often linked to the isolation of an area from networks which we take for granted, including the internet, electric grids, gas mains and water facilities. An area which is without these and without the means to build them tends to become more and more cut-off from the hyper-centers and consequently from any and all economic activity. The possibility of creating an autonomous agricultural system could reduce rural exodus, often linked to insufficient water provision for agriculture, and hence

the problems that go with massive emigration on the one hand, and immigration on the other. Problems linked to emigration include a collapse of the local economy, brought on by an insufficient labor supply. This is particularly true when you take into account the fact that the demographic faction that is most likely to leave is young people, who are also the strongest workers. Immigration can cause over-taxation of a city's resources which the authorities do not have the means to deal with. This can be linked to the formation of ghettos and an inefficient waste treatment system which in turn are linked to the spread of disease. Brain drain can also lead to a flooding of the labor markets in certain areas, which can threaten workers' rights through the increase in the number of people competing for a single job. From a Keynesian point of view, a decrease in wages and hence spending power can lead to an economic slump. In contrast, the decrease in rural exodus is linked to the development of areas that are currently marginalized or even forgotten, thus curbing a phenomenon which is a serious economic drain.

In conclusion, we do not for a moment doubt that in the face of increasing deregulation and water scarcity, the characteristics of *hydrocoli* or similar water treatment biotechnologies will make it the most efficient choice in terms of costs, public health, sustainability and safety. We hope that in this perhaps small way we may contribute to a safer and more egalitarian future.

VII.1 CONCLUSIONS

Our proposed goal was to show how issues stemming from synthetic biology were taking showing up in various fields. We hope to have reached this goal. The field of synthetic biology remains a closed one, and the details are still mysterious for a large portion of the public. However, thanks to international events where synthetic biology takes center stage, such as IGEM, it is becoming more familiar to people outside the field. As we have seen, there are numerous issues, more every day, that must be resolved. This process is difficult but society as a whole must reach for the light at the end of the tunnel.

The birth of a new scientific discipline and its road towards becoming commonplace must be dealt with responsibly in order for humanity to benefit without causing damage to the environment. This must include education, and, more importantly, consultation, of the general public because it is consumers who ultimately make a technology successful or not. Researchers, as well as the guardians of bio-safety and bio-security, must also take responsibility to ensure that uncontrolled and dangerous dissemination of these technologies is not possible. Just as certain plants have both healing and dangerous properties, synthetic biology can benefit humanity, or damage humanity if misused. Our position is neither alarmist nor utopian; in fact, a happy medium between technophobia and

technophilia must be struck, especially when dealing with such highly complex matters as those concerned by synthetic biology.

We firmly believe that this technology has the power to do good, all the while bringing significant financial returns to investors. This can happen if, and only if, those responsible for its commercialization and implementation proceed with caution. On the one hand, from a human point of view, caution must be used in the face of ethical concerns, safety and the economic analysis of humanitarian aid. While they must be addressed, these concerns will not even have the opportunity to see the light of day if the product is not successfully commercialized. From a commercial point of view, care must be taken to avoid the pitfalls of negative consumer attitudes and culture shock, leading to the rejection and hence the failure of the innovation.

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