iGEM 2007 Wiki
International Genetically Engineered Machine Competition

# The SMB: Synthetic Multicellular Bacterium

The aim of our project is to engineer the first synthetic multicellular bacterium, the SMB. This new organism is a novel tool for the engineering of complex biological systems. It consists in two interdependent cell lines. The first, dedicated to reproduction is the germ line (red cells in the simulation below). It is able to differentiate into the second line: the soma (green cells), which is sterile and dedicated to support the germ line. The germ line is auxotroph for DAP (diaminopimelate) which is provided by the soma. There is thus an interdependency relationship. The soma, being sterile, requires the germ line for its generation, while the germ line needs the soma to complement its auxotrophy. We provide here both experimental and computational evidences that this system can work, as well as the almost complete construction of the SMB.

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# 1. Paris/Introduction From IGEM07

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## 1.1Why a Synthetic Multicellular Bacterium ?

### Dealing with complexity

A major challenge for synthetic biology is to tackle complexity *(Simpson 2004)*. If one wants to modify an organism so that it can perform complex tasks, the standard approach is to implement a complex circuit within a single cell. But unwanted interactions may happen between parts of the system, either because of unknown or unavailable interactions. An alternative approach may be to implement small simple parts of your circuit within different types of cells that would work together.

For the moment only very few parts in the registry are well characterized. This leads to a situation in which almost all the devices are made out of the same biobricks (plac, pBad, ptet, plambda, luxI, luxR…), and are thus incompatible (you cannot implement them in the same cell). Implementing them in different cell types could alleviate this problem…

The aim of our project is to provide a practical tool for such an approach, making a jump in complexity scale, from single cells to multicellular synthetic biology, and for the first time engineering a **multicellular bacterium**.

### A new tool for metabolic engineering

In most multicellular organisms, there is a distinction between the cell line devoted to reproduction, the germline, and the one devoted to support the germline, the soma (*Weismann, 1892*). The particularity of somatic cells is that, in a sense, they sacrifice themselves for the benefit of the organism. In the same way, implementing a soma/germ line separation in a bacterial species could allow using the soma to produce

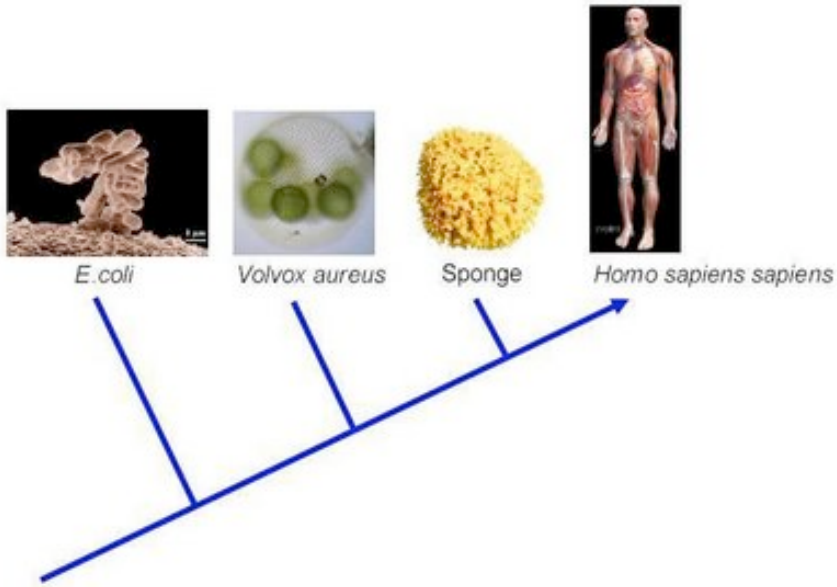
compounds noxious for itself. In this perspective, our multicellular organism could become a great tool for metabolic engineering, and to synthetic biology at large.

### Studying fundamental features of multicellularity

A separation between germline and soma is a basic feature of multicellular organisms. Although the fitness of the organism essentially depends on the phenotype of the soma, only the germline is perpetuated. For instance skin cells die for the sake of the organism. Our SMB could be a phenomenological model to study the relationship between those two cell types.

## 1.2 What is a multicellular organism and what is needed to create one ?

### Grouping of cells




Organisms are living complex adaptive system composed of cells. Some organisms, such as bacteria or protozoa, are unicellular. Other organisms, such as humans, are multicellular. But providing a clear definition of multicellularity is not obvious.

*E. coli* is a bacterium able to form colonies but is a unicellular organism since a single cell is able to survive on its own if separated from the colony. Colonial organisms, such as *Volvox*, are considered being an evolutionary intermediate between the unicellularity and multicellularity, living in colonies most of the time, even if individual cells can survive on their own if separated from the colony (click here to know more about the colonial theory of evolution of multicellularity). Another example is the one of recently discovered Magnetobacter that seem to necessarily live in colony (*Keim, 2004*). Consequently, it can be discussed whether the grouping of identical cells constitute a multicellular organism.

Since there is no consensus on the subject, our design of a synthetic multicellular organism will include two cells types and we **will define a multicellular organism as the necessary and sufficient existence of at least 2 cell types**.

## Cellular differentation

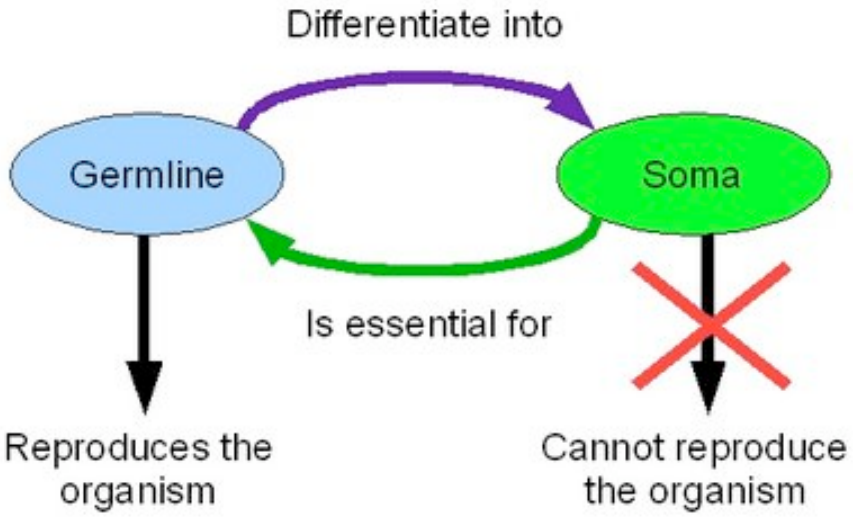
A basic property of multicellular organisms is the occurrence of cellular differentiation leading to complementary specialized functions and interdependency between different cell types. Cellular differentiation is the process by which a cell acquires a new cell type. A cell type is a distinct functional and/or morphological form of a cell. A cell able to differentiate into many cell types is known as pluripotent. Generally, differentiation leads to progressive restriction of the developmental potential and increased specialization of function (NCBI MeSH).

Differentiation needs signalling between and within cells leading ultimately to changes in gene expression patterns: some genes are turned on and other are turned off. Considering how gene expression may change, we can distinguish 2 forms of differentiations. Most of the time, it is epigenetic differentiation where mechanisms do not involve changes to DNA sequence: "classical" cis and trans gene regulation involving transcription factors, enhancers, repressors but also DNA methylations, DNA replication timing... The second form of differentiation involves changes to DNA sequence by recombination processes, for example during lymphocyte differentiation (V-D-J recombination; *Tonegawa, 1976*).

## Germ cells and somatic cells

In multicellular organisms, two categories of cells can be found. Germ cells are responsible for the reproduction of the organism while the others, the somatic cells, are unable to generate a new organism but are essential for the germ line viability. Most multicellular organisms reproduce through sex, and the germ line is responsible for gamete production. We will not attempt to reproduce sexual behaviors, only separation between a line dedicated to reproduction, and one dedicated to support the other will be implemented. These will be named the germ line and the soma. Also, we realize we are overextending the proper definition of those terms.

## 1.3 What will our synthetic multicellular organism look like?




Our organism, is be composed of 2 cell types. The first one is the germ cell (G cell). It is unable to live alone but able to give an entire organism (both cell types). It needs the second cell type to survive: the somatic cell. Somatic cells (S cell) are derived from G cells by a single irreversible differentiation step and concomitantly acquire a new function necessary for supporting G cells survival. Therefore, the S cell is unable to give an entire organism.

Thus, our system is composed of an undifferentiated G cell type and a differentiated S cell type. Obligatory interdependency between these two cell types must ensure the stability of the system. The nature of this interdependency will be discussed below.

## 1.4 References

1. Keim, C. N., J. L. Martins, et al. (2004). "Multicellular life cycle of magnetotactic prokaryotes." FEMS Microbiol Lett 240(2): 203-8.

2. Simpson, M. L. (2004). "Rewiring the cell: synthetic biology moves towards higher functional complexity." Trends Biotechnol 22(11): 555-7.

3. Tonegawa, S. (1976). "Reiteration frequency of immunoglobulin light chain genes: further evidence for somatic generation of antibody diversity." Proc Natl Acad Sci U S A 73(1): 203-7.

4. Weismann, A. (1893). The Germ-Plasm - A Theory of Heredity. New York, Charles Scribner's Sons.

# 2.Paris DesignProcess From IGEM07

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## 2.1 SMB Design Process

The first question that needs to be addressed in the design process of our system is that of the nature of the dependency relationships between its cellular components. Namely, what cross-dependency relationship between the somatic & germ line cells can best ensure “ecological” equilibrium & “evolutionary” stability between the two cell types?

### The soma needs the germ line

#### *What dependency relationship?*

A radical mean of ensuring somatic cell dependence on germ line is to make somatic cells sterile. That is, if somatic cells are unable to replicate, then they exclusively depend on G=>S differentiation for their existence. Furthermore, in this way it is impossible for S cells to overtake the population.

How can sterility be achieved?

A great number of essential genes have been identified since the early days of E.coli genetics. An essential gene is one for which a mutant strain cannot grow at 37° on rich medium (LB). These genes include the essential division genes. Mutants of these genes cannot divide. Suppressing the expression of such a gene would thus make the cell sterile.

Differentiation mechanism

As discussed above, differentiation can be epigenetic (changes in genes expression pattern) or genetic (change in DNA sequence). In order to suppress the expression of an essential gene, we could either place it under the control of an inducible promoter that we would repress (epigenetic differentiation), or remove the gene itself through recombination (genetic differentiation). A genetic mechanism of differentiation is more robust than an epigenetic one in a way that the system artificially implemented in the cell would escape with more difficulties.

Therefore, we decided that the Germline=>Soma differentiation mechanism should be genetic (as opposed to epigenetic). Sterility in S cells will be achieved by deleting an essential gene from the genome during the differentiation process. This will be done through site-specific recombination.

Constraints on essential gene choice

Remains the question of the choice of the essential gene to be used. We have decided to base our choice on two criteria:

- **Longevity of the S cells:** S cells even if unable to divide should be able to live as long as possible. (an explanation for this is given below)
- **Gene isolation:** In our final construct, cassettes will be inserted before and after the essential gene. We want our gene to be isolated, so as not to disturb the expression of other nearby genes. It should not for instance be embedded in an operon. See here for more details about the construction method.

We have finally chosen ftsK as the gene to be used. It is an isolated gene. Also, ts (thermo sensitive) strains for this gene give filamentous cells at the restrictive temperature that continue growing for an equivalent of over 10 generations resulting in impressively long cells.

# The germ line needs the soma

## *What dependency relationship?*

In our synthetic organism, the germ line is auxtroph for a given nutrient that is provided by the soma. In this way, a part of the germ line needs to differentiate, in order to feed the undifferentiated fraction.

*Why an auxotrophy dependence?*

Previous works have shown that two types of cell, each auxotroph for a different metabolite, can rescue each other when grown in the same minimum media. This means that some auxotrophy should be rescued in a coculture with prototroph cells. Plus, it is very easy to do!

## What auxotrophy?

In order to choose our auxotrophy, we need to take several constraints into account:

- **No simple bypass or reversion:** The auxotrophy phenotype of the germ line must be stable. There must be no simple metabolic bypass or reversion possibilities.
- **Overproduction and excretion:** Prototroph cells must be able to overproduce and excrete the metabolite, or at least to release it when dying.
- **Absorption:** auxotroph cells should be able to live in very low concentration of this metabolite. Indeed we should expect only little metabolite to be excreted.
- **Survival:** auxotroph cells should be able to live as long as possible when deprived from the metabolite.
- **No growth on LB:** We would like our synthetic organism to be able to grow on rich medium. This would facilitate our lab work, and more generally allow our synthetic organism to grow in a variety of conditions.
- **Feedback:** For purposes described below, we would like to have a sensor device sensitive to our metabolite concentration.

We finally choose diaminopimelate (DAP) as our metabolite. The germ line will be deleted for the dapA gene, an essential gene in the peptidoglycan and lysine biosynthesis pathways. For details about our choice :

**What metabolite should we use for the feeding of the germline by the soma ?**

In order to choose our auxotrophy, we need to take several constraints into account:

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**Candidate metabolite DAP:**

Diaminopimelate is a precursor both for lysine and for cell wall components. It is synthesized from aspartate-semialdehyde, through 5 reactions catalysed by (DapA, DapB, DapD, ArgD and DapE). All this five genes are essential, that is to say, when any of these genes is deleted the strain is DAP auxotroph and cannot grow on rich medium.

It as been found that mutants of the gene catalysing the step from DAP to lysine (LysA) are excreting DAP (1). This is supposedly because of the negative feedback loop of the lysine concentration on the DapA activity. This provides a proof that DAP can be excreted by the cell when overproduced. In order to reproduce this effect, we can try to find DapA mutants which would be insensitive to feedback. The lysine pathway is pretty well conserved across all kingdoms. In plants, DAP is only an intermediated in the lysine biosynthesis. There is no constitutive need for DAP, and there is a strong negative feedback of lysine on DapA. On the other side, gram+ bacteria which have a thick cell wall, present a strong need for DAP without respect to the lysine concentration. Gram+ DapA genes are thus insensitive to lysine. Gram– bacteria which have a smaller cell wall, also have a smaller need for DAP, which is consistent with the fact that there is a small negative feedback from lysine on gram- DapA.

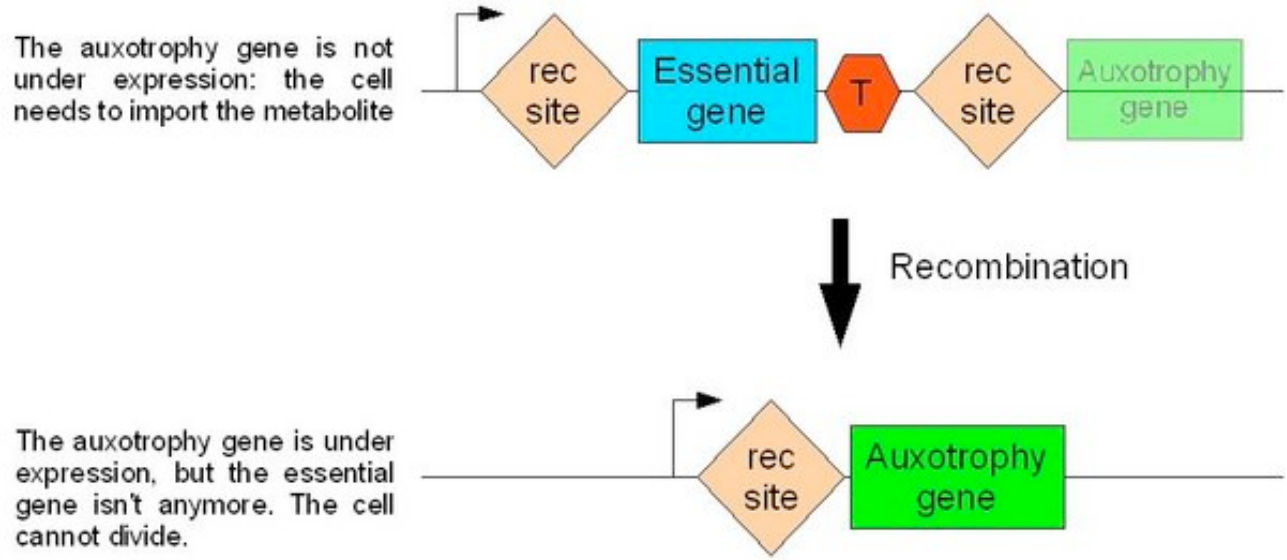
Soy DapA feedback insensitive mutants have been identified and expressed in Coli, showing complementation of DapA mutants and DAP overproduction (2). We can also think of using gram + DapA genes.

Last point, DapA expression has been shown to depend on DAP concentration (3). The mechanism is unknown, but there is great chance that any gene placed under the control of the DapA promoter will have its expression controlled by DAP concentration.

A particularly appealing feature of dapA for our system is that some bacterial species have DapA enzymes that are not submitted to allosteric inhibition by DAP. The activity of E.coli DapA protein is to some extent inhibited by DAP. However, Bacillus subtilis DapA enzyme is not submitted to such a control. This enables the possibility of using B.subtilis dapA gene in our system for DAP overexpression.
In order to confirm that our choice fits well the above mentioned criteria, we conducted several experiments. (See the results page)

## *How will auxotroph germ line cells differentiate into prototroph somatic cells?*

We found an elegant way to solve this problem. This is best described by the following schema.




rec is a site specific recombination sequence

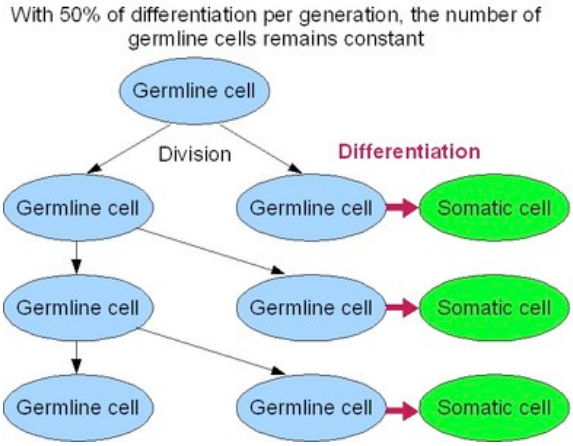
T is a strong terminator

## What differentiation rate?

Our system's success relies on the differentiation rate of the germ line into soma. An intuitive reasoning shows that we cannot have more than 50% of differentiation per generation. Otherwise, the germ line will only decrease. But this is not the only constraint to take into account.

For our germ line to grow fast, we need to maximize the DAP production. This means that we need to maximize the excretion of DAP by the soma and to maximize the proportion of the soma itself. The number of somatic cells is given by the differentiation rate of germ line cells and the life time of somatic cells. Thus we need to maximize the **life time of somatic cells** and the **differentiation rate** of the germ line. On the other hand, we want our synthetic organism to grow as fast as possible. And it will grow faster if the proportion of germ line cells is bigger, since somatic cells do not divide. This means that we have to minimize the differentiation rate.

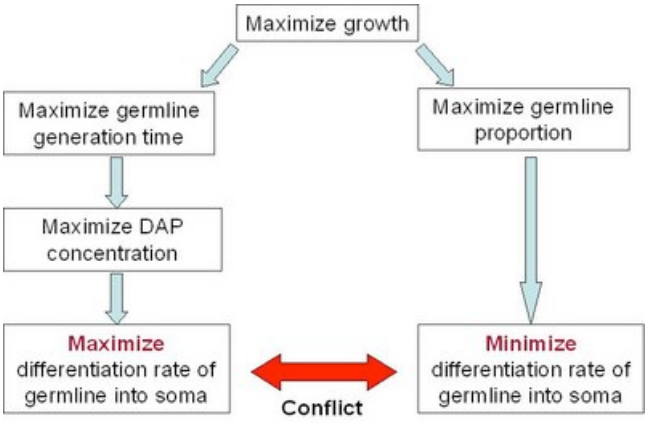
We clearly see the conflict here, which will ultimately lead to a trade-off between the germ line proportion and its generation time. As explained above, the differentiation rate cannot be over 50% per generation if we want our synthetic organism to grow. What this trade-off suggests is that there is an optimum differentiation rate somewhere between 0 and 50% recombinant per generation. The effect of the differentiation rate on the growth rate is displayed in the modeling of our system.




## *What recombination system?*

According to the previous point, we need a recombination system whose rate can be controlled. Ideally we wish to obtain recombination at any chosen frequency. If this is impossible, we need at least the recombination rate to be below 50% per generation, but high enough for the germ line to be sufficiently fed. Another constraint on the recombination system, is that we want differentiation to be unidirectional. We thus chose the Cre/Lox recombination system, for the following reasons:

- it is readily available, largely used and well described.
- lox66 and lox71 recombination sites have been described to produce unidirectional recombination *( Cre recombinase-mediated inversion using lox66 and lox71: method to introduce conditional point mutations into the CREB-binding protein, Zuwen Zhang and Beat Lutz, Nucleic Acids Research)*




We want to control the recombination frequency by adjusting the expression level of the Cre recombinase. To do this, we cloned the Cre recombinase under the control of the pBad promoter (inducible through arabinose). We also decided to clone our Cre production device (araC-pBad>>rbs-Cre) on a low copy number plasmid, to broaden the reachable expression rates.

Recombinaison frequency measurment experiments

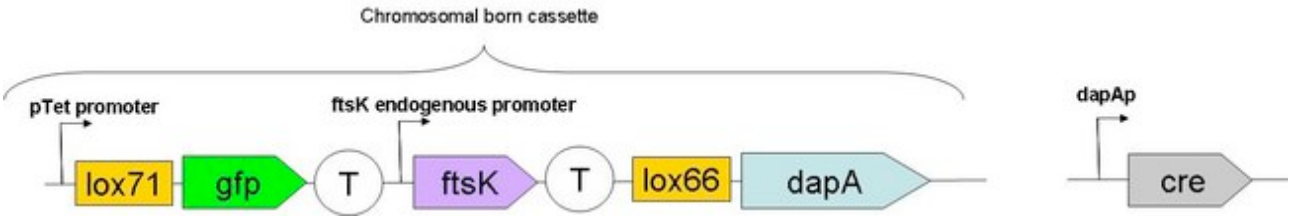
## *Optimization through feedback*

It is clear that the average recombination rate must be between 0 and 50% per generation. Nevertheless, we can theoretically maximize the growth rate by adjusting the recombination rate to DAP level. The quantitative analysis on the population model investigates this. In this way, the germ line would increase its differentiation rate upon DAP starvation, and reduce it when there is enough DAP. In order to achieve this, Cre

expression must be adjusted to DAP concentration. This would be easily done if there where a promoter sensitive to DAP concentration, which seems to be the case of the dapA promoter (dapAp). We therefore cloned and characterized this promoter.

## Overview of the project

After going through the system design process, we have selected a number of basic features to be included in the SMB system. A cassette, functional when inserted in the chromosome, needs to be generated.



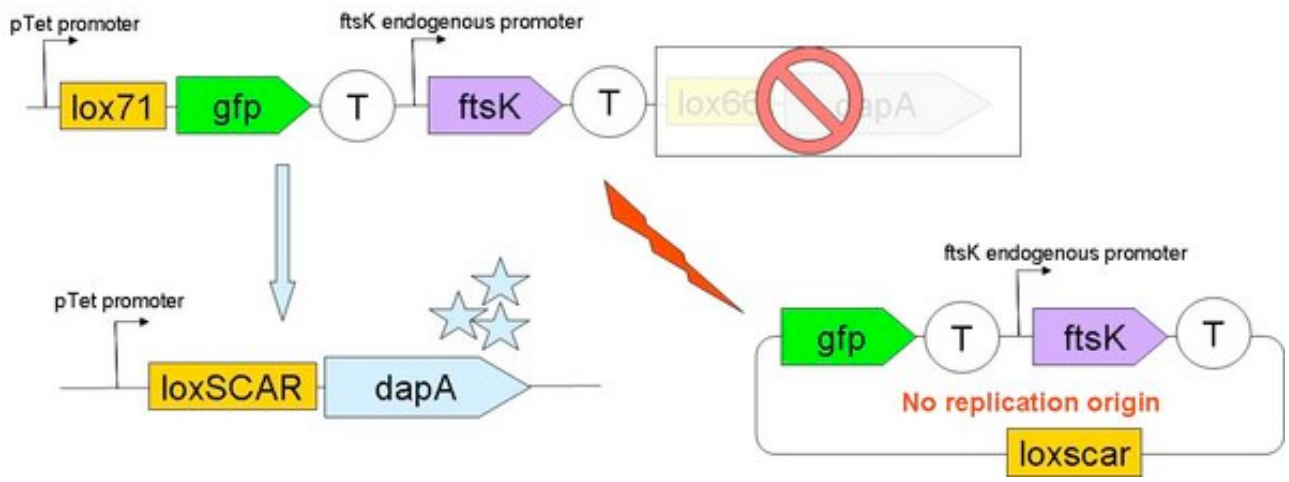

This full construct is in process of insertion into the genome.

As can be seen in this schematic representation of the SMB genomic backbone cassette, at basal genomic state (in Germ line cells, before recombination):

- pTet promoter drives the expression of gfp.
- The expression of ftsK is controlled by its natural promoter.
- ftsK is isolated from pTet promoter by the intercalation of Terminator (B0015 terminator).
- dapA gene is in a dormant state since it lacks a promoter to drive its transcription.

To learn more about the construction process, click here or follow the link at the end of the page.

Upon G to S differentiation, the following genomic reassembly should take place:




Cre mediated lox recombination should lead to:

- excision of ftsK gene from the genome onto a circular non-replicative DNA molecule. We ignore how much time such a DNA is stable in a cell.
- placing dapA gene under the control of pTet promoter. This should lead to dapA expression, & hopefully to DAP synthesis.

# *E. Colight*: towards a new slim diet

## Project

Triglycerides are composed of a molecule of glycerol esterified by three fatty acids. When ingested, triglycerides are hydrolysed by lipases, in the stomach and the duodenum, into glycerol and free fatty acids. Enterocytes are can only absorb free fatty acids and glycerol. These are subsequently recombined in the cytoplasm into triglycerides. Triglycerides are then freed in the lymphatic system and then in the blood within fatty vesicles called chylomicrons.

When lipid uptake versus energy consumption and loss is unbalanced, we accumulate fat. Knowing that gut is full with bacteria forming the gut microflora (we have $10^{13}$ cells in our body and $10^{14}$ bacteria with the majority in the gut!), we envision engineering bacteria capable of absorbing fatty acids and storing them in the form of triglycerides intracellular inclusion. These triglycerides would not be absorbed by enterocytes! Lipid input would decrease! Eat fat, don't get fat!

In fact, a drug, orlistat, already exists and shows decreasing lipid input works in obesity. It comes from a bacterial lipase inhibitor (from Streptomyces toxytricini). It inhibits pancreatic lipase and is used to cure obese people and type 2 diabetes with hypocaloric cure. At the standard prescription dose of 120 mg three times daily before meals, orlistat prevents approximately 30% of dietary fat from being absorbed (Thomson

PDR, 2006).

E.coli is the most used bacterium in synthetic biology and... belongs to the gut microflora! We wish to genetically engineer E. coli into Ecolight to store triglycerides into inclusions! Knowing that 40% of E.coli is renewed every day, these triglyceride-filled bacteria will leave the gut with faeces!

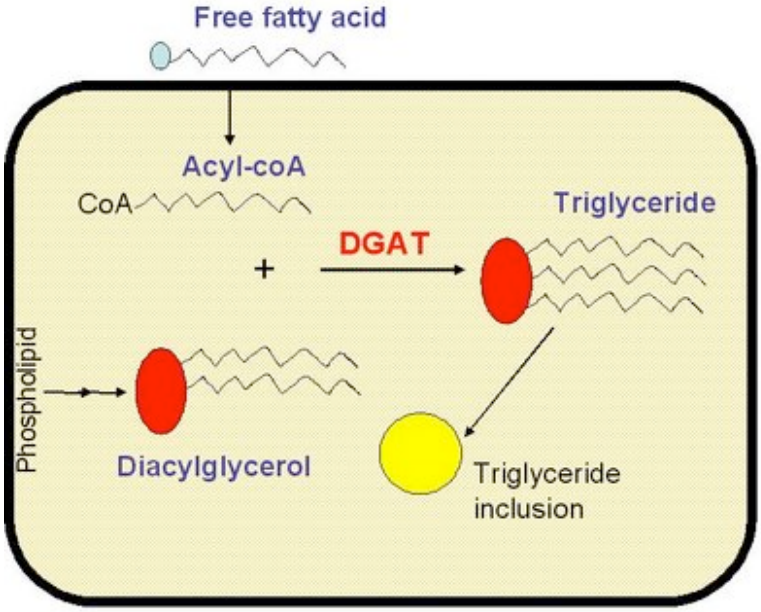
The result of this work should, in a second phase of our work, be combined with the "security device" derived from the SMB described above. This is further discussed in PERSPECTIVES.

## Technical details

As a perspective for our SMB, we want to show that it can be a tool for metabolic engineering (see perspectives). We started to develop the idea of making E. Coli store fatty acids in the form of triglycerides. Such an engineered bacteria could be ingested to absorb fatty acids thus limiting the amount of fatty acids absorbed by the user! Eat fat don't get fat !

- **Why should it work?**

Triglycerides are not a natural product of E. coli metabolism as it lacks diacylglycerol acyl-transferase (DGAT). DGAT catalyses the reaction of glycerol esterifaication by fatty acids. However, all the compounds necessary for the triglyceride synthesis are present in the cytoplasm: diacylglycerol is an intermediate of phospholipid catabolism (source ecocyc.org) and free fatty acids are imported from extracellular medium. Wild-type E. coli strains can indeed grow on free fatty acid medium (oleate for example). E. Coli has a Long Chain Fatty Acid (LCFA) transporter, FadL. Another protein: FadR, a long chain acyl-CoA-responsive transcription factor, controls the expression of nine genes primarily involved in fatty acid degradation and biosynthesis (FadL is thus induced by FadR) and β-oxidation enzymes.



- **Which enzyme?**

We decided to use DGAT enzyme imported from a bacterium closely related to E. coli for metabolic compatibility. Acinetobacter calcoaceticus ADP1, a Gram negative bacillus was a good candidate. Its DGAT enzyme is also an acyl-CoA fatty alcohol acyltransferase (wax ester synthase) and catalyzes the final condensation of acyl-CoA and fatty alcohol. But knowing that E.coli does not produce fatty alcohol, this reaction is probably not avaible. (for more information see BBa_I718002).

# Design Process References

(1) *Relation Between Excreted Lipopolysaccharide Complexes and Surface Structures of a Lysine-Limited Culture of Escherichia coli* K. W. KNOX,' MARET VESK,2 AND ELIZABETH WORK

(2) *Soybean DapA mutations encoding lysine-insensitive dihydrodipicolinate synthase* Gregg W. Silk and Benjamin F. Matthews

(3) Acord04: Acord J, Masters M (2004). *"Expression from the Escherichia coli dapA promoter is regulated by intracellular levels of diaminopimelic acid."* FEMS Microbiol Lett 235(1);131-7. PMID: 15158272

# 3. Paris Modeling From IGEM07



## 3.1 Introduction

### Motivation for Modeling

We want to construct a multicellular bacterial organism made of two co-existing cell types. Brainstorming resulted in proposing the following system:

- so-called soma cells, that produce a metabolite (DAP), and will not be able to divide,
- so-called germ cells that are able to grow, but only in presence of (sufficient quantities of ) DAP, and are able to differentiate into soma cells.

Informal reasoning indicate that this design should be correct, in the sense that it leads to *an exponential growth of the two coexisting cell types*. However, before actually constructing this system, we would like to assess the quality of our design using modeling approaches. Since different questions needed to be answered, we developed different types of models, *each adapted to a particular problem*.

## Questions of Interest

The first, most obvious question deals with proving the *feasibility* of our system ( Section 2). In our case, this amounts to check that the system presents a very simple, qualitative behavior: *the two cell populations grow*! Accordingly, we tested whether this property holds under various modeling assumptions.
The simplest model that we have considered is a phenomenological ODE model ( Section 2.1). Being very simple, analytic analysis is possible and the stability of equilibria can be investigated under mild assumptions on parameters. However, a number of phenomena that might play an important role are neglected in this model. By assuming that cellular and molecular concentrations evolve continuously and that the solution is well-mixed, noise and space-related issues may have been overlooked. To test whether these phenomena may affect the qualitative behavior of the system (i.e. growth), we developed two models, one focusing on spatial aspects of Dap diffusion on cell differentiation ( Section 2.2), the other incorporating dynamical aspects of cell spatial organization (Section 2.3). These results are rather general, in the sense that the level of abstraction of these models does not allow to distinguish between the two slightly different designs proposed in section DESIGN PROCESS:

- A SMB with a fixed recombination rate
- A SMB with a recombination rate depending on DAP starvation (feedback)

In addition to feasibility, robustness and tunability of the system are also of prime interest. More precisely, we would like to find an objective criteria to discriminate between the two competing designs proposed in section Design process. The two designs differ by the presence or absence of a negative regulation of cre recombinase expression by Dap. To address this problem, we developed two numerical ODE models and investigated their relative robustness and tunability ( Section 3.1). Finally, it is also important to check that stochastic phenomena that are neglected in ODE models do not affect the macroscopic behavior. Stated differently we checked whether the deterministic models and their stochastic counterparts present globally the same behavior (Section 3.2).

## 3.2 Proof of Principle: Qualitative Analysis of System's Behavior

In this section, we develop models to test the feasibility of our system. We focus on a simple, essential qualitative property: the growth of the two coexisting cell types. This property is investigated under various modeling assumptions.

### *3.2.1 Exponential growth of cellular populations: analytic analysis of an ODE model*

We present here a theoretical approach based on population dynamics. We consider here the case of a well mixed, homogeneous, culture of the SMB organism, i.e. there is no space in this analysis and we follow only the variation of the different cell lines concentrations in the culture volume.

**Derivation of the model**

Let the variables g, s and d describe respectively the concentrations of germinal and somatic cells and the concentration of DAP, then we can write for our system:

$$\dot{g} = \alpha_1 \frac{d^n}{k^n + d^n} g - \alpha_2 g - \alpha_3 g \quad (1a)$$

$$\dot{s} = \alpha_2 g - \alpha_4 s \quad (1b)$$

$$\dot{d} = \alpha_5 s - \alpha_6 d \quad (1c)$$

Equation (1a) describes the growth of the germinal cell population in presence of sufficient DAP (interaction represented by the Michaelis-Menten function) ; the term proportional to α2 is the differentiation into somatic cells by recombination of the CRE/LOX box, and the last term proportional to α3 is the germinal cells' death. Equation (1b) is the variation of the somatic cells' population, with the term proportional to α4 for somatic cells' death. The last line describes DAP production by the somatic cells, and includes a degradation term. In absence of any quantitative details on assimilation of DAP by germinal cells and response to DAP levels, n and k are are to be considered as arbitrary phenomenological parameters. We take however in the following n = 1 neglecting potential saturation related non-linearities for high DAP concentrations. The value of k corresponds to the DAP concentration for half-maximal growth rate, and could set experimentally. We simplify the previous system by assuming that the evolution of d is rapid compared to the cellular growth, so that at this time scale we can take d' = 0 and write d = s α5/ α6 . This gives the two-variable system:

$$\dot{g} = \left(\alpha_1 \frac{s^n}{\left(\frac{k\alpha_6}{\alpha_5}\right)^n + s^n} - \alpha_2 - \alpha_3\right) g \quad (2a)$$

$$\dot{s} = \alpha_2 g - \alpha_4 s \quad (2b)$$

Redefinition of parameters k → kα6 /α5 and α3 → α2 + α3 leads to the simpler writing:

$$\dot{g} = \left(\alpha_1 \frac{s^n}{k^n + s^n} - \alpha_3\right) g \tag{3a}$$

$$\dot{s} = \alpha_2 g - \alpha_4 s. \tag{3b}$$

Let us do some rewriting:

$$\frac{dg}{d(\alpha_3 t)} = \left(\alpha_1 \frac{\left(\frac{\alpha_3}{\alpha_2}\right)^n s^n}{k^n + \left(\frac{\alpha_3}{\alpha_2}\right)^n s^n} - 1\right) g \tag{4a}$$

$$\frac{d\frac{\alpha_3}{\alpha_2} s}{d(\alpha_3 t)} = g - \frac{\alpha_4}{\alpha_3}\frac{\alpha_3}{\alpha_2} s, \tag{4b}$$

and by redefining the time and most of the parameters we get:

$$\dot{g} = \left(\alpha \frac{s^n}{k^n + s^n} - 1\right) g \tag{5a}$$

$$\dot{s} = g - \beta s \tag{5b}$$

The fixed points are (g = 0, s = 0) and the solution of:

$$(\alpha - 1) s^n = k^n \tag{6a}$$

$$g = \beta s, \tag{6b}$$

**Analysis of stability**

*Fixed point at the origin*

Let us linearize the system (5a) close to the origin. For small perturbations around (g = 0, s = 0) (5a) is equivalent to :

$$\begin{pmatrix} \dot{g} \\ \dot{s} \end{pmatrix} = \begin{pmatrix} -1 & 0 \\ 1 & -\beta \end{pmatrix} \begin{pmatrix} g \\ s \end{pmatrix} \tag{7}$$

We look for a solution of the form $\vec{B}e^{\lambda t}$, with $\vec{B}$ an arbitrary vector. The values of λ are the eigenvalues of the matrix in (7) and can be obtain here straightforwardly by the characteristic polynomial : λ1 = –b, λ2 = –1, with respectively the eigenvectors $\vec{V_1} = [0,1], \vec{V_2}[1, \frac{1}{b-1}]$.

The two eigenvalues are always negative : the origin is therefore always an attractive fixed point. For too weak initial concentrations of the two cellular types, the system is always going to die out.

*Second fixed point, out of the origin*

Let (g0 := β s0 , s0 := k/(α–1) ) be the non trivial solution of (6a). We can simplify the (5a) by dividing the two equations respectively by g0 et s0 and by taking as new variables G := g/g0 and S := s/s0:

$$\frac{d}{dt}\left(\frac{g}{g_0}\right) = \frac{\alpha\frac{s}{s_0} - \frac{k}{s_0} - \frac{s}{s_0}}{\frac{k}{s_0} + \frac{s}{s_0}} \cdot \frac{g}{g_0} \quad (8a)$$

$$\frac{d}{dt}\left(\frac{s}{s_0}\right) = \beta\left(\frac{g}{g_0} - \frac{s}{s_0}\right), \quad (8b)$$

and with μ := α–1 = α1/α2– 1 :

$$\frac{d}{dt}G = \frac{\mu(S-1)}{\mu+S}G \quad (9a)$$

$$\frac{d}{dt}S = \beta(G-S) \quad (9b)$$

The fixed point is now (G0=1,S0=1). We linearize system (9a) around this point and look for a solution close to it. Take x := G–G0 and y := S–S0 , close to (G0,S0) we can write by keeping only the first order term of (9a) :

$$\begin{pmatrix}\dot{x}\\ \dot{y}\end{pmatrix} = \begin{pmatrix}0 & \frac{\mu}{1+\mu}\\ \beta & -\beta\end{pmatrix}\begin{pmatrix}x\\ y\end{pmatrix} \quad (10)$$

Looking for a solution of the form $\vec{B}e^{\lambda t}$, we need to find the eigenvalues of the matrix in (10). Solving the characteristic polynomial gives :

$$\lambda_1 = -\frac{1}{2}(\beta + \sqrt{\beta^2 + 4\frac{\beta\mu}{1+\mu}}) \quad (11)$$

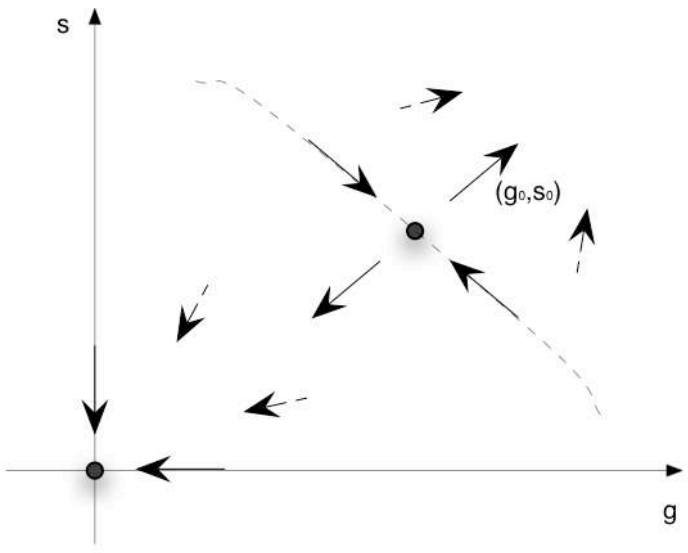
$$\lambda_2 = -\frac{1}{2}(-\beta + \sqrt{\beta^2 + 4\frac{\beta\mu}{1+\mu}}), \quad (12)$$

In order to determine the stability of the fixed point, let us examine the signs of the eigenvalues : λ1 is always negative, while λ2 is positive if μ/1+μ > 0. That is, by returning to the original parameters of (1) if α1 > α2 (more growth than recombination). In this case (which is expected) the fixed point (G0, S0) is unstable and the populations of the two cell lines diverge.

**Conclusion**

If we put together the results on the two fixed points we get the situation represented on the following diagram :

For a region around the origin and below some line passing through (G0,S0) any initial conditions converges to the origin. For higher values of the initial conditions we always expect exponential growth of the populations. Indeed, if in the unstable case, we extrapolate the asymptotic behaviour of our system, (2), we expect both population to grow, at some point the Michaelis-Menten term saturates, and equation (2a) becomes g' = (α1-α2-α3)g. The solution is a exponential with a positif exponent in this case.




It is interesting for comparaison with experimental measurements to evaluate the asymptotic behaviour of the ratio of the growth rate over population size g'/g. For this discussion and without loss of generality in the treatment (implies only a redefinition of the parameter α2 ) we neglect cell death, we will also keep n = 1 for the reason mentioned before. From the preceeding paragraph we see that tha population of g cannot grow for α1 = α2 , that is when the time for cell division (α1)^(-1) is equal to the generation time of somatic cells (α2)^(-1) . Both cell types have the same growth rate, in our system it means that for each germinal cell division there is one recombination of the

CRE-LOX box and the generation of one somatic cell. This means that we need less than 50% recombination probability in order for our system to work. For recombination rates α2 (for fixed α1 ) close to this limit, the growth rate over population size curve as a function α2 is a straight line with negative slope g'/g = α1 – α2 , going to zero at 50% recombination probability.

### *3.2.2 Potential macroscopic effect of spatial aspects of Dap diffusion: cellular automaton on a grid*

In this part of our work, we aim at characterizing the diffusion of the DAP and the effect on the cells differentiation. This study consists in observing by simulation, the diffusion of DAP in a lawn of germ cells with some isolated somatic cells using a cellular automaton.

**Introduction**

DAP feeding between somatic and germ cells is based on an indirect communication process: soma cells produce DAP and release it in the environment; DAP molecules freely diffuse outside until they are captured by a germ cell. As the diffusion takes place in the environment, a somatic cell feed first the germ cells that are close to it. Then an well mixed hypothesis as the one used in the our growth of population analysis is hard to assume. We are interesting here in the case where the differentiation of a germ into a soma is DAP dependent. In order to figure out the relation between DAP diffusion and differentiation we propose a simple cellular automaton on square grid. Each cell of the automaton contains a bacterium. We first detail some hypotheses used in this model, then we specify the local behavior rules following by each automaton cell. Finally, the generated simulation is presented.


*Hypotheses*

The chosen approach consists in observing the DAP diffusion and differentiation frontwaves. In order to focus on these phenomenon, we work on a *constant* population (no death, no division). So we assume that without DAP in its surrounding, a germ cell does not die but remain in passive state (we can imagine that they are at a stationary phase or between to division cycle). It will seem that DAP *wake up* bacteria but it's just an artifact due to this assumption.

It may happen that a germ cell as enough DAP to evolve (typically when it is touched by a DAP diffusion front) but we assume that the contribution is not enough for the cell to divide.

Finally, we assume then that DAP is produced in somatic cells only and consumed by germ cells. The communication is done by distinguishing in the automaton intra and extra cellular DAP (respectively named DAPi and DAPe).

*Model Description*

In this we focus on the elaboration of the cellular automaton.

*Structure*

As we have previously announced, we design cellular automaton on a square grid. More precisely, in order to avoid boundary effects, we assume that the grid is actually wrapped in such a way the grid topology is a 2D torus. Each cell of the automaton contains a bacterium, either germ or somatic, together with the external DAP concentration. So we represent the different states of the automaton cell by tuple of values `{DAPe,DAPi,Type}`:

- `DAPe` is the external DAP concentration,
- `DAPi` is the internal DAP concentration in the bacterium,
- `Type` represents if the bacterium is differentiated or not; it can take two values `BactG` and `BactS`.

*Dynamics*

The following rules specify the local evolution of each cell of the automaton. We distinguished to evolution laws depending on what kind of bacterium is in the cell:

- In the case of a somatic cell: we have to consider the diffusion of `DAPe` between the considered cell and its neighbors, the export of DAP from the inside to the outside, and finally the production of `DAPi`. The rule can be presented as follows:

```
DAPe <- DAPe + (DAPe diffused in the neighborhood) + (DAPi lost by export)
DAPi <- DAPi + (DAPi produced) - (DAPi lost by export)
Type <- BactS
```

- In the case of a germ cell: we have to consider the diffusion of `DAPe` between the considered cell and its neighbors, the import of DAP from the outside to the inside, the consumption of `DAPi`, and finally the differentiation when DAP concentration reaches a right range of values. The rule can be presented as follows:

```
DAPe <- DAPe + (DAPe diffused in the neighborhood) - (DAPi gain by import)
DAPi <- DAPi - (DAPi consumed) + (DAPi gain by import)
Type <- if (min_threshold) < DAPi < (max_threshold) then BactS else BactG
```

*Parameters*

We consider 8 parameters. They are used with some noise during the evolution to avoid a deterministic behavior.

- In `BactS` cells:
- Dap export rate in somatic bacteria
- Dap import rate in somatic bacteria
- Dap production rate of somatic bacteria
- In `BactG` cells:
- Dap export rate in germ bacteria
- Dap import rate in germ bacteria
- Dap consummation rate of germ bacteria

- Minimal threshold for differentiation
- Maximal threshold for differentiation

## *Initial state*

Our initial state is 30x30 2D toric cellular automaton where all cells are initialized by value `{DAPe=0,DAPi=0,Type=BactG}` but four `{DAPe=0,DAPi=0,Type=BactS}` are randomly placed in the grid.

## *Output*


Our implementation was done in MGS and the output was generated by GBView.

The output is two animated pictures: the first one shows the differentiation, the other the diffusion of DAPe

*(See animation on the wiki)*

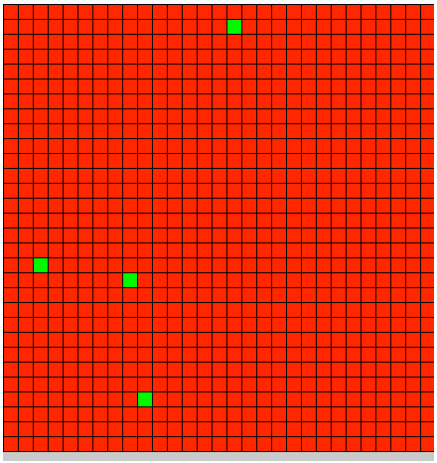
- The first picture shows the diffusion of DAP: the front wave is figured in light blue; the dark blue area corresponds to stable parts of the system where concentration do not evolve anymore.
- The second picture presents the differentiation: red and blue cells are respectively germ and somatic bacteria.

**Results**

As we can see, the differentiation and DAP diffusion wave fronts are superposed. This simulation is obviously out of reality but the underlying model have been developed in order to consider that low concentration of DAP induces differentiation (cells become green - dark blue), while with high DAP concentration, the differentiation is inhibited. Without this second threshold, all cells would differentiate. But some germ cells remain because they are enough fed to stay over the threshold. This property is of course crucial as only germ cells can reproduce.

In the considered model of the system, the inhibition must be strong and effective for not all the cells differentiate. Bad thresholds make the system collapse: a too strong inhibition prevents germ cells to differentiate, and on the opposite, a too weak inhibition make all them switch to a somatic state.

By tuning the constant of diffusion, we have noted that the 3 steps communication process (export, diffusion, import) is of main interest. For a few molecules of DAP imported in germ cells, an important amount of produced DAP has to be produced: there is a lot of lost during the process. So in order to keep a coherent rate of production, a germ cell must be surrounded by a lot of somatic bacteria: the ratio of 1:1 of dif-



ferentiated and germ cell is not viable, that is why a lot of somatic cells feed isolated germ bacteria as enlighten by the animations.

Sources: http://parts.mit.edu/igem07/index.php/Paris%5CSources#Cell_auto

### *3.2.3 Potential macroscopic effect of stochastic and spatial aspects of Dap diffusion and cell growth*

In this section, we aim at considering SMB as a dynamical system with a dynamical structure and studying the impact of the cells organization on the future of the population. In order to achieve this goal, we have developed a mechanistic model based on a masses/springs system, that will allow cell to divide and die.

**Introduction**

The cellular automaton develop to compare diffusion of DAP and differentiation is very restrictive and unrealistic due to the lake of natural behavior of cells like growth, division, death ... As a matter of fact, a cellular automaton point of view does not allow to deal with dynamical population:

- required automaton rules to push cells and to allow divisions, are hard to carry out;
- the rigid structure of the grid prevent any topological modification of the population organization;
- somatic cells that cannot divide keep on growing and become significantly bigger that germ bacteria, a missing notion in a cellular automaton.

With this second simulation we focus on this issue. We consider SMB as a dynamical system with a dynamical structure and aim at studying the impact of the cells organization on the future of the population. In order to achieve this goal, we need a mechanistic model that will allow cell to divide and die. We propose to use a masses/springs model. Such model allow

- division and death by adding or removing masses,
- cell growth by increasing springs rest length,
- to fill holes in the population (if there is some empty place in the population springs will push masses to fill it),
- to prevent a dispersion of cells (springs cannot infinitely extends).

This mechanistic model has to be coupled with the biological one we previously have developed.

We first stress out the hypothesis of this simulation, we then detail the mechanistic and biological models, and finally, we simulate the system considering either a DAP controlled differentiation or a DAP independent differentiation.

**Hypotheses**

In this model we made four important hypotheses:

- Even if the somatic cells feed germ ones by an indirect process (using an external diffusion in the environment and not a direct bridge communication between cells) we consider that DAP cannot diffuse very far from a somatic cell and then a somatic cell can only feed close (in fact neighbor in our model) germ cells. As a consequence, we choose to model a direct communication (equivalent to an indirect communication with a small DAP diffusion rate) by putting a black box on the underlying complex mechanisms of feeding. In other words, there is no distinction between external and internal DAP; exchange are done directly from cell to cell.
- We consider that the population evolves in size. So we allow bacteria to die and divide. Of course, with respect to initial system, somatic cells cannot divide. On the other, we assume in this model that germ cells cannot die. In fact, we are here in-

terested in studying the growth of the system that compulsorily requires presence of germ cells.
- This point is not really an assumption but an property of cells that was not taken into account in the previous models. We will consider that cells are growing in size. In fact, the size of a somatic cell is supposed to be bigger than standard germ size. This could disturb a lot the geometric organization of the population.
- Finally the two following case will be considered:
- First case: the differentiation is DAP dependent
- Second case: the differentiation is done with a constant rate

**Description of the Model**

As we have already said, this model is divided into two:

- the mechanistic model that is used to deal with the cells displacement (using mechanical constraints) and their neighborhood,
- the biological model that make cells evolve, growth (by extended springs rest length), divide, die, differentiate and communicate.

Both are mainly independent (the only dependence appears with the growth where springs length are modified). So we will present them separately.

**The Mechanical Model**

**Neighborhood**

In the mechanistic part of the system, a cell is considered as a punctual mass localized in the 2D euclidean space. All the cells are sharing the mass (we choose 1 to simplify computation). Cells are also characterized by there velocity. Finally, in order to compute the spring rest length, each cell exhibits its radius. We represent them by a tuple `(px,py,vx,vy,r)` where `px` and `py` are the coordinate of the cell position, `vx` and `vy` are the coordinates of its velocity vector, and `r` represents its radius.

The neighborhood between cells is computed using a Delaunay triangulation. In our system, if the triangulation make two cells become neighbors, this means that a spring is considered between their corresponding mass. The rest length of this spring is then the sum of the radius component of the mass representation. Once again to simplify computation the force constant of the spring is 1.

This characterization of the cells as masses corresponds to the definition of type `MecaBact` in the program. Fields have been added to save the sum of the forces that act on a mass.

**Dynamics**

We compute the displacement of a mass during a small time step by a Euler approximation. At each time step, the forces applied on each mass are summed, and twice integrated to compute the velocity, then the displacement and finally the new position of the mass. The considered forces are of two types: the restoring force exerted by the springs on each mass, and a friction force to prevent a divergent increase of the velocity and to ensure that the mechanical equilibrium will be reached.

The dynamics have implemented using the transformation `Meca`.

**The Biological Model**

*Structure*

The biological cells are represented in the same way as in the previous cellular automaton. Each bacterium is either germ or somatic and is characterized by its DAP concentration. So we represent the different states of the automaton cell by tuple of values `{DAP,Type}`:

- `DAP` is the internal DAP concentration in the bacterium,
- `Type` represents if the bacterium is differentiated or not; it can take two values `BactG` and `BactS`.

The neighborhood is given by the mechanical model.

This characterization of the biological cells corresponds to the definition of type `CellBact` in the program.

*Dynamics*

The biological dynamics are really simple:

- somatic cells produce DAP, grow and die
- germ cell consume DAP, grow, divide and differentiate
- DAP is diffusing between cells

These rules are specified in the transformation `Evol`. In this transformation two rules are defined the first one to deal with somatic cells evolution and the second one with germ cells evolution. These rules can be understood as follows:

- For somatic cells `(DAP,BactS,r)`:

```
DAP <- DAP - (DAP production) - (Diffused DAP)
if (random < probability of death)
then
   kill the bacterium
else
  if (random < probability to grow) & (r < maximum cell size)
  then
     r <- r + delta
```

- For germ cells `(DAP,BactG,r)`, the evolution depends on the DAP dependent or independent differentiations model
- Case 1 (DAP dependent)

```
DAP <- DAP - (DAP consummation) - (Diffused DAP)
if (random < probability of differentiation) & (enough DAP) <- Here is the
DAP dependence
then
   Type <- BactS
else
  if (r > maximum cell size)
  then
     if (random < probability to divide) & (DAP > minimal DAP required for
division)
    then
      divide
```

```
   else
     if (random < probability to grow)
     then
       r <- r + delta
```

- Case 2 (DAP independent)

```
DAP <- DAP - (DAP consummation) - (Diffused DAP)
if (random < probability of differentiation) <- No DAP dependence
then
   Type <- BactS
else
  if (r > maximum cell size)
  then
     if (random < probability to divide) & (DAP > minimal DAP required for
division)
     then
       divide
  else
     if (random < probability to grow)
     then
       r <- r + delta
```

## *Coupling models*

Finally, considering together the two models, a cell is represented by a tuple `(DAP,Type,px,py,vx,vy,r)`.

The dynamics are simply coupled by composing functions `Meca` and `Evol`. See function `step`.

## *Parameters*

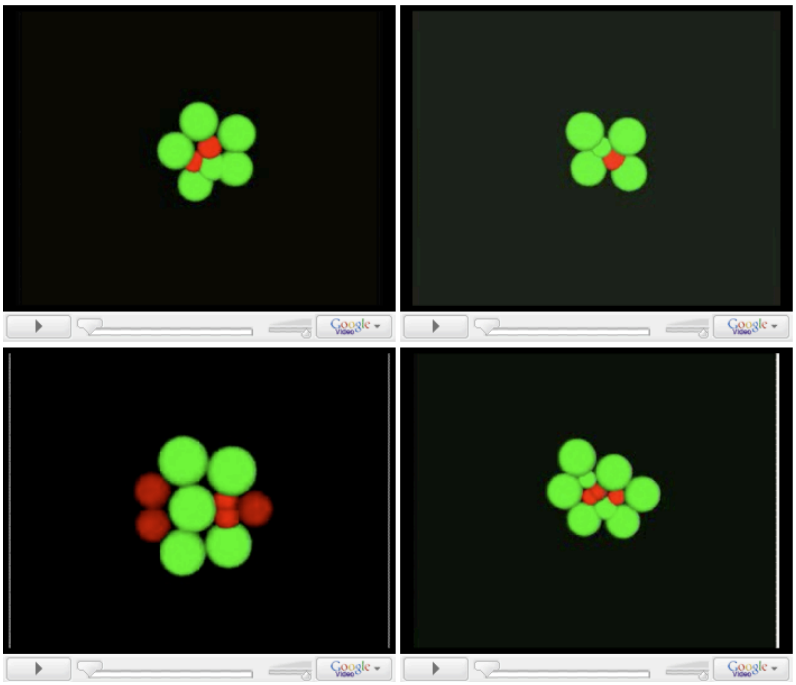
As it appears in the previous algorithms, a lot of parameters can be used to obtain different kind of evolutions. We introduce the reader to the 8 parameters:

- `DT`: the time step. The same time step is used in the integrations of the mechanical model and in the DAP diffusion of the biological model. An application of function `step` computes the evolution of system during after `DT`.
- `K` and `Mu` are respectively the spring constant (all the springs share the same constant) and the friction constant.
- `R0_Gm`, `R0_G` and `R0_S` are the different threshold defining the range of cells radius. They are respectively the minimal germ cell size (after division), the maximal germ cells size (before division), and the maximal somatic cells size.
- `Diff` characterized the DAP diffusion rate.
- `Diffp` is the probability of differentiation.
- `DEPOT` is the constant DAP production of somatic cells per unit of time.
- `CONS` is the constant DAP consummation of germ cells per unit of time.
- `DeathSP` is the probability of death of somatic cells.
- `DivGP` is the probability of division of germ cells.

- `CroitS` and `CroitG` are respectively probability of growth of somatic and germ cells.

**Results**

Thanks to MGS and ImoView (a software dedicated to the 3D visualization of MGS programs outputs. The input of ImoView are written in a symbolic high-level scene description languages.) we are able to generate the following animations.



*(See animations on the wiki)*

In these animation, red and green spheres respectively represent germ and somatic cells. The brightness variations of the germ cells indicates the evolution of DAP concentration: the darker the color is, the lower the concentration is.

On the first line, both animations are DAP **dependent** simulations, while the bottom line presents two animations of DAP **independent** simulations. Both models (with and without dependence) qualitatively exhibit the same global behavior. Only the speed of the population growth differs; the system seems to be more efficient when a DAP dependent differentiation is considered.

The columns show another property of the system that is predicted in the phenomenological study: it exists an unstable equilibrium. On the left, the behavior corresponds to evolving organism that develops itself and colonizes the environment: that is the exponential growth of the population. On the right, parameters have been tuned to simulate a stable evolution like in a tissue or an organ. The fact that this fixed point is unstable is here verified: due to the stochastic choice done in the model, using the same set of parameters that produce right column, the population sometimes collapses in simulations, falling in the trivial fixed point.

Sources http://parts.mit.edu/igem07/index.php/Paris/Sources#Cell_auto_2

## 3.3 Assessing Robustness and Optimizing System's Behavior: Quantitative Analysis

In this section, we focus on more quantitative properties of system's behavior: robustness and optimization capabilities. Two slightly different designs are compared.

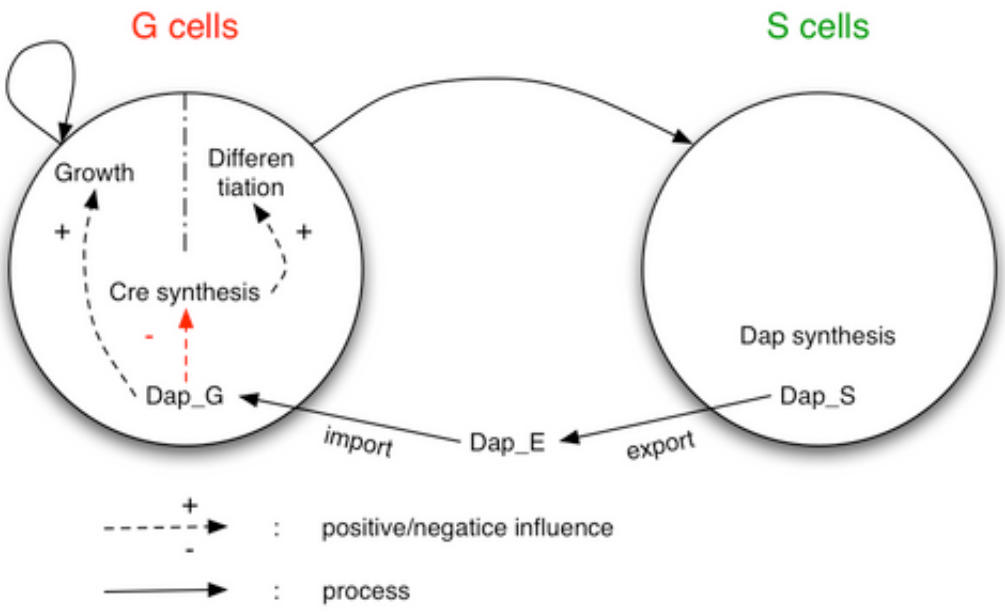
### 3.3.1 Assessing robustness and tunability of two potential designs: numerical simulations of ODE models

In section Design process two designs have been proposed. The only difference is that one of them incorporates a negative feedback of cre recombinase by dap. We developed simple models to evaluate the relative benefits of both designs in term of robustness and optimization capabilities.

#### Introduction

This model aims at assessing robustness of the system with regards to kinetic parameters and initial condition variations and at investigating the possibilities to tune the system to improve its behavior. Additionaly, we would like to compare the robustness and optimization capabilities of two slightly different designs, one with a constant rate of synthesis of cre recombinase and one with a rate of synthesis driven by the concentration of Dap.
This approach differs from the first one by the level of description of the model and the numerical analysis done on the model. More precisely, it is based on a set of differential equations describing Dap synthesis, Cre synthesis, Dap transport, differentiation of germen bacteria into soma and bacteria death.
We first provide a set of kinetic parameters such that the numerical simulation validate a given minimal behavior. Then we analyze the system robustness with regard to variations of its kinetic parameters and initial condition and finally we investigate ways to optimize the system behavior by adjusting some biologically relevant parameters.

### *System*



The system studied is made of two populations of bacteria G (germen) and S (soma). Bacteria G can either divide or differentiate into S.

Dap is only synthesized by bacteria S and diffuses in the environment and in G.

G is dependent of Dap for division. Differentiation of G is controlled by Cre. Cre synthesis in G is either constant or dependant of Dap (red arrow).

## *Expected behavior*

We consider initial conditions in which only G cells are present. Because both Dap and S cells are absent, the system has to go through an intialization phase in which G differentiates into S cells and S cells start producing Dap. This initialization phase is vulnerable since no cell growth is possible. Adding Dap in the environment can only have a benefic effect on the population so we choose to start this study with an initial condition lacking Dap.

In summary the expected behavior for this system is that cell populations grow fast enough and have a robust initialization phase.

## *Problems*

- Is minimal behavior robust ?
- How can we tune the system to improve its behavior ?
- Which design should we prefer ?

# *Modelling*

**Differential equations**

$$(1) \qquad \frac{d[G]}{dt} = \lambda_{div} \cdot [G] \cdot \frac{[Dap^G]^{\eta_{Dap}^{div}}}{\theta_{Dap}^{div\ \eta_{Dap}^{div}} + [Dap^G]^{\eta_{Dap}^{div}}} - \lambda_{diff} \cdot [G] \cdot \frac{[Cre]^{\eta_{Cre}^{diff}}}{\theta_{Cre}^{diff\ \eta_{Cre}^{diff}} + [Cre]^{\eta_{Cre}^{diff}}} - \lambda_{death} \cdot [G]$$

$$(2) \qquad \frac{d[S]}{dt} = \lambda_{diff} \cdot [G] \cdot \frac{[Cre]^{\eta_{Cre}^{diff}}}{\theta_{Cre}^{diff\ \eta_{Cre}^{diff}} + [Cre]^{\eta_{Cre}^{diff}}} - \lambda_{death} \cdot [S]$$

Equations (1) and (2) takes into account cell division, differentiation and death. To model the dependence of division on Dap and differentiation on Cre for G cells we use Michaelis-Menten kinetics (terms $\lambda_{div}$ and $\lambda_{diff}$ in equations 1 and 2 with $\eta_{Dap}^{div}=\eta_{Cre}^{diff}=1$).

$$(3) \qquad \frac{d[Cre]}{dt} = \kappa_{Cre}^{leak} + \kappa_{Cre} \cdot \{\frac{\theta_{Dap}^{Cre\ \eta_{Dap}^{cre}}}{\theta_{Dap}^{Cre\ \eta_{Dap}^{cre}} + [Dap^G]^{\eta_{Dap}^{cre}}}, 1\} - \gamma_{Cre} \cdot [Cre]$$
$$+ \lambda_{div} \cdot \frac{[Dap^G]^{\eta_{Dap}^{div}}}{\theta_{Dap}^{div\ \eta_{Dap}^{div}} + [Dap^G]^{\eta_{Dap}^{div}}} \cdot [Cre]$$

Equation (3) represents the synthesis of Cre, its degradation and its dilution caused by Gcells growth. We consider two models : the synthesis rate of Cre is either constant (design 1) or inhibited by Dap (design 2) as indicated by the red term. Term $\lambda_{div}$ in equations (3,4) accounts for the dilution of Cre and Dap when the population of G grows.

$$(4)\quad \frac{d[Dap^E]}{dt} = \kappa_{exp}\cdot[Dap^S]\cdot[S] + \kappa_{exp}\cdot[Dap^G]\cdot[G] + \lambda_{death}\cdot[Dap^S]\cdot[S] + \lambda_{death}\cdot[Dap^G]\cdot[G] - \kappa_{imp}\cdot[Dap^E]\cdot[G] - \kappa_{imp}\cdot[Dap^E]\cdot[S] - \gamma^E_{Dap}\cdot[Dap^E]$$

$$(5)\quad \frac{d[Dap^G]}{dt} = \kappa_{imp}\cdot[Dap^E] - \kappa_{exp}\cdot[Dap^G] - \gamma^S_{Dap}G\cdot[Dap^G] - \lambda_{div}\cdot[Dap^G]\cdot\frac{[Dap^G]^{\eta^{div}_{Dap}}}{\theta^{div}_{Dap}{}^{\eta^{div}_{Dap}} + [Dap^G]^{\eta^{div}_{Dap}}}$$

$$(6)\quad \frac{d[Dap^S]}{dt} = \kappa_{Dap} + \kappa_{imp}\cdot[Dap^E] - \kappa_{exp}\cdot[Dap^S] - \gamma^S_{Dap}G\cdot[Dap^S] + \lambda_{diff}\cdot\frac{[Cre]^{\eta^{diff}_{Cre}}}{[Cre]^{\eta^{diff}_{Cre}} + \theta^{diff}_{Cre}{}^{\eta^{diff}_{Cre}}}\cdot([Dap^G]-[Dap^S])\cdot\frac{[G]}{[S]}$$

Equations involving Dap (4,5,6) represent Dap synthesis in soma bacteria, Dap degradation in bacteria and in the environment, Dap transport and the release of Dap in the environment from dying bacteria. Term $\lambda_{diff}$ in equation (6) is a corrective term to take into account the mean variation of concentration of Dap in G and S when some bacteria G differentiate into S.

## *Parameters*

We first try to find out a set of parameter values such that the system exhibits a minimal growth and a robust initialization phase. We will then use this set of parameter values as a reference point for the robustness and optimization analysis.

### *Initial condition*

The initial conditions correspond to a situation where only G cells are present, that is no bacteria S, no Dap and no Cre.

### *Kinetic Parameters*

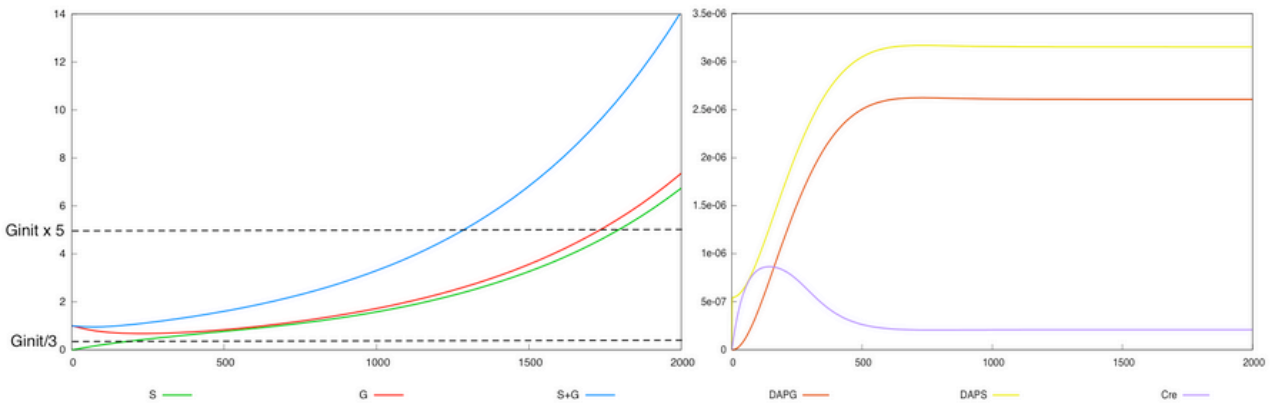
In order to set parameter values we use several different methods. First, parameters λdiv and λdeath are estimations based on experimental data. Then, we fix inequality constraints expressing on the one hand biological knowledge : Dap is actively imported into bacteria (κimp > κexp), bacteria death is lower than bacteria growth (λdeath<λdiv), degradation of DAP in the environment is lower than in bacteria where it is consumed (γDapE < γDapGS),transport of DAP is an order of magnitude faster than reactions modelling death, growth or differentiation of bacteria (κexp,κimp>> λdiv,λdeath,λdiff) and on the other hand intuitive reasoning : differentiation must be lower than bacteria growth (λdiff > λdiv).

Based on these constraints on the parameters, we defined a set of parameter values p* as a reference starting point.

Specification
We define a minimal behavior that the system must display. First, we want that the quantity of bacteria G never falls below threshold Ginit/3, where Ginit is the initial G cell population. Secondly, we want that after a certain amount of time (2000 time units), the overall population of bacteria (G+S) has grown enough, that is, is above threshold 5*Ginit.

We use the modeling environment BIOCHAM to model, simulate and check whether the system validates this specification, which is expressed by the temporal logic formula G([G]>Ginit/3) & F<2000([G]+[S]>5*Ginit). This litterally express in temporal logic that the amount of G cells ('[G]') is always ('G') above threshold Ginit/3 and ('&') that the population ('[G]+[S]') reaches in less than 2000 time units ('F<2000') a threshold 5*Ginit'.

For the parmameter values p*, the two possible designs validate this specification. The system with a synthesis rate of Cre dependent of Dap (Design 2) has the following behavior :




**Analysis**

## *Robustness*

In order to evaluate the robustness of both models, we try to find a box in the parameter space containing p* such that for any point of this box the specification we defined is valid.

First of all, we reduce the number of relevant parameters to ease the enumeration process. Several parameters values act by pair, such has $\kappa_{exp}/\kappa_{imp}$ determing the ratio DapE/DapGS. For each of this pairs we retained only one parameter. By using this method we extracted 9 parameters from the existing 19 parameters.

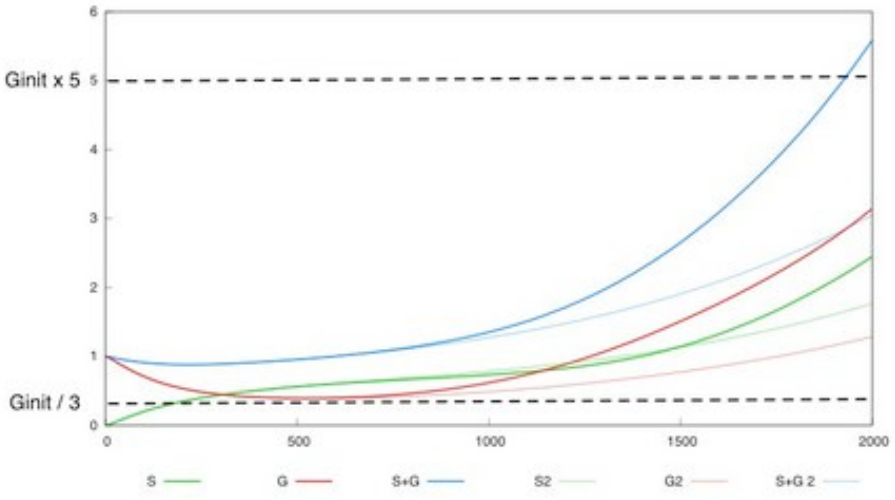
Then, to find the box we begin by searching for a box such that for any vertex of this box the system is valid, that is making 2^9 simulations and checking for everyone whether the specification is valid. Once such a box is found we sample the inner area to check if the specification is valid in inside the box. We sampled each parameter's interval value with 4 points. It takes about 9 hours to make simulations for 4^9 different parameter values.

For the system with a variable rate of synthesis of Cre the following box is valid :

| | $\lambda_{diff}$ | $\lambda_{div}$ | $\lambda_{death}$ | $\kappa_{exp}$ | $\theta_{div}^{dap}$ | $\theta_{diff}^{cre}$ | $\theta_{cre}^{dap}$ | Ginit |
|---|---|---|---|---|---|---|---|---|
| % decrease | 20 | 1.5 | 90 | 50 | 20 | 8.6 | 30 | 10 |
| % increase | 10 | 4.6 | 10 | 100 | 10 | 100 | 15 | 1000 |

The system is very responsive to parameters λdiff, λdiv and λdeath. A combined very small change of these 3 parameters in the system is enough to invalidate the required behavior.

The second system with a constant synthesis rate of Cre is not valid in this whole box. 536 out of the 262000 points in this box are not valid for this system. We show a simulation of the system's behavior for the two different designs (thin lines for design 1, thick lines for design 2) and for the same set of parameter values, inside the box. For the system with unregulated cre synthesis (design 1) the expected porperty is not satisfied.




This tends to show that *having a negative feedback of Cre synthesis by Dap leads to a more robust design*.

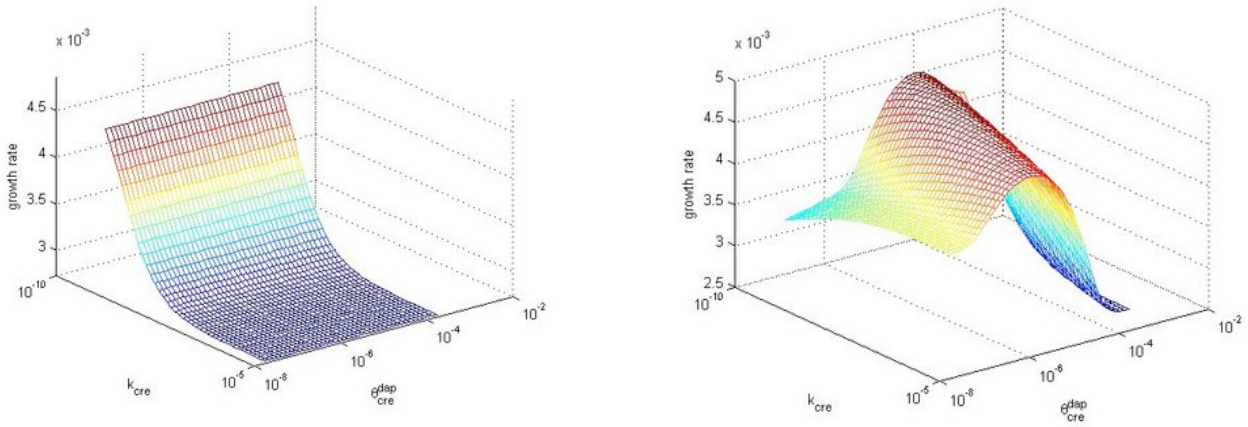
### *Optimization*

We compare the growth of the system to the growth of the 'equivalent' wild type bacteria (same growth and death rates). After 2000 time units, the wild type bacteria attains a population of 61000 compared to 14 when parameter values are set to p*. Our SMB organism grows very slowly!

For that reason we try to adjust some parameter values to obtain a better growth. We only consider parameters *directly corresponding to biologically adjustable properties* of the system. These parameters are cre synthesis rate ($\kappa_{cre}$), dap synthesis rate ($\kappa_{dap}$), and inhibition constant of cre synthesis by dap ($\theta_{cre}^{dap}$). Moreover we consider we can change, *i.e.*, increase or decrease, these parameters at most by two orders of

magnitudes. Our approach is to sample this interval values for each parameter to find the optimal combined value of these parameters in this range.

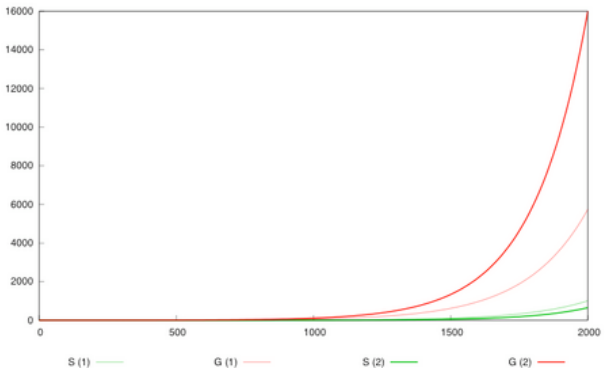
Below, the growth rate of the population is represented as a function of cre synthesis rate ($\kappa_{cre}$) and of cre inhibition constant ($\theta_{cre}^{dap}$) for the two possible designs (design 1, cre regulation, left and design 2, no cre regulation, right). All other parameters have the same values in the two models. These graphs illustrate that a higher growth rate can be achieved in design 2 than in design 1. Moreover, the second design offers better capabilities to tune the system, sonce one can increase growth rate by playing either with cre synthesis rate or with the affinity of cre for the dap promoter.

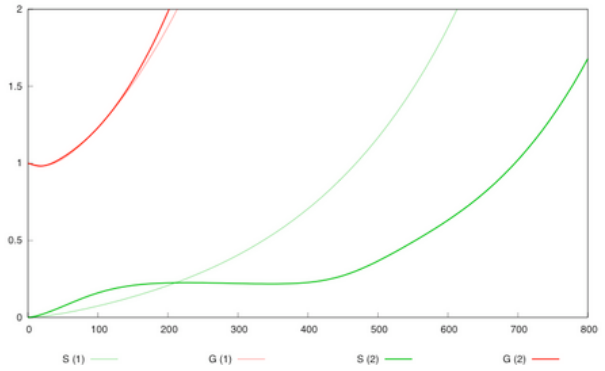


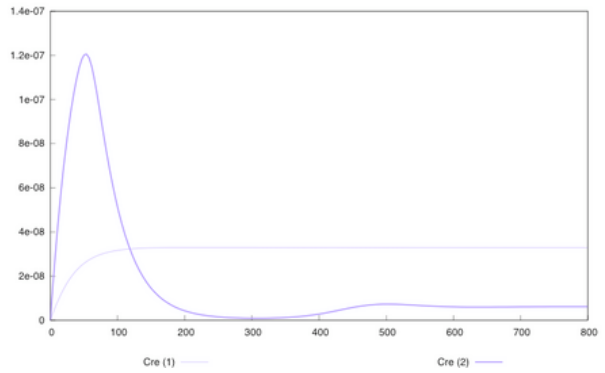

The population exhibits an exponential growth of type exp(kt). In order to measure the relative growth of each set of parameters we compare value k in the exponential. The wild type has a growth k=λdiv-λdeath=0.055. For p*, the apparent growth is exponential with k=0.013. The best value for design 2 (with cre regulation) is 0.049 and is obtained for κcre=κcre* /20, κdap=κdap* *100 and θcredap=θcredap* * 4. For design 1 (no cre regulation) the best value is 0.044 and is obtained for κcre=κcre* /100, κdap=κdap**100.

It is important to note that in both cases, the maximal growth is obtained for the highest value of the DAP synthesis rate (κdap). This has been true in all our complementary tuning analyses (not shown). This motivates our choice of the B.subtilis dapA gene. As explained in the Design process section contrary to E.coli, in B.subtilis the protein is not inhibited by DAP. This could increase DAP production rate, and consequently the population growth.

Here are the simulation obtained for these values in both designs :




First curve displays the evolution of populations G and S in 2000 time units, the second one the evolution of G and S in 800 time units (initialization phase) while the third one shows off Cre concentration in 800 time units.

First, we notice that the regulated system has a better start : high Cre concentration leads to a rapid increase in S cells and thus to higher dap amounts and a better growth of G cells. After initialisation phase is over the regulated system is still better : inhibition of Cre synthesis allows a lower Cre concentration, a lower differentiation rate that is a higher G/S ratio and a better growth.

This tends to show that *having a negative feedback of cre synthesis by Dap leads to a system having better optimization capabilities*.

**Summary**

These results tend to indicate that the model with negative feedback loop on Cre synthesis presents an increased robustness.

In order to have a system with a population growth rate similar to the growth of the wild type, S cells have to produce a lot of Dap and differentiation of G cells has to be low so that ratio S/G << 1. Optimization anlysis shows that it is easier to achieve this with the regulated system. Inhibition of Cre synthesis by Dap indeed allows to have a very low overall differentiation in the growth phase and still have a fast initialization phase thanks to the initial burst of Cre caused by the lack of Dap in the initial condition.

## 3.3.2 Potential macroscopic effect of stochastic phenomena: stochastic simulations with Gillespie algorithm

In this last part of the models section, we are developing a stochastic simulation of the microscopic model. The major contribution is to handle a dynamic and heterogeneous population of bacteria in a stochastic context.

In 1977, Gillespie developed an exact Stochastic Simulation Algorithm (SSA) dedicated to the simulation of homogeneous chemical systems. This method was recently used in many applications for the simulation of biological systems. A good point of this approach is that it allows to handle biochemical systems where numbers of molecules are low and that cannot be well characterized by classical approaches using differential equations and chemical concentrations. Nevertheless this method requires strong hypotheses about the spatial homogeneity of molecules distribution. Extensions of Gillespie's SSA have been proposed to deal with compartments.

As our system is composed of a growing and heterogeneous population of bacteria, we propose to use this extension to simulate it. In the following paragraphs, we first detail the extended SSA we use and then we present some samples generated by our implementation using the set of parameters found in the numerical analysis of the model. Note that the main contribution here is in the development of the simulation algorithm.

### *Extended SSA*

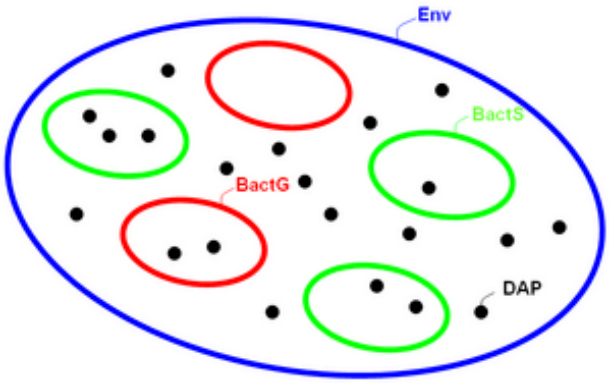
#### Gillespie's SSA

From a computational point of view, the Gillespie SSA relies on a discrete events simulation of chemical reactions between individual molecules. A reaction Rmu like A + B -> C occurs when reactants A and B meet with enough energy to produce a molecule C. The probability that this reaction occurs during an infinitesimal time, is proportional to the number of molecules A and B in the system (dependence on the concentration) and a coefficient cmu called the stochastic constant (corresponding to the reaction kinetic) when reactants are uniformly distributed in the system (intuitively it means that each couple of molecules has the same probability to meet). Assuming a system composing of molecules that can interact with respect to reactions R1 ... RN, this probability allows to compute the probability law p(mu,tau) of the next reaction to occur is Rmu after tau units of time.

The algorithm developed by Gillespie consists in iterating the drawing of the next reaction mu to occur together with its reaction time tau using p(mu,tau), and in making the system evolves. Assuming that S is chemical solution where reactions R0 ... RN can occur at a given time t, the SSA can be expressed as follows:

```
SSA (S,t,R0,...,RN) = {
  compute mu and tau using p(mu,tau)
  compute S' by applying mu on S
  return  (S',t+tau)
}
```

## Handling Membranes




In the SSA, molecules have to be uniformly distributed in space. It cannot be straightly used to deal with systems that exhibit a complex spatial organization, as the system we are interested in. Taking space into account means modifying the probability law p(mu,tau) in order to take care of the localization of the reaction. Obviously, it cannot be computed in practice for any kind of organization, but it is possible to develop ad-hoc algorithms for specific organizations.

We propose to consider a population of bacteria as a nested membranes system, called compartments. The environment where bacteria live is represented by a compartment that contains molecules and bacteria, and a bacterium is then represented by a compartment that contains molecules. In each compartment, the contained elements are assumed to be uniformly distributed in space.

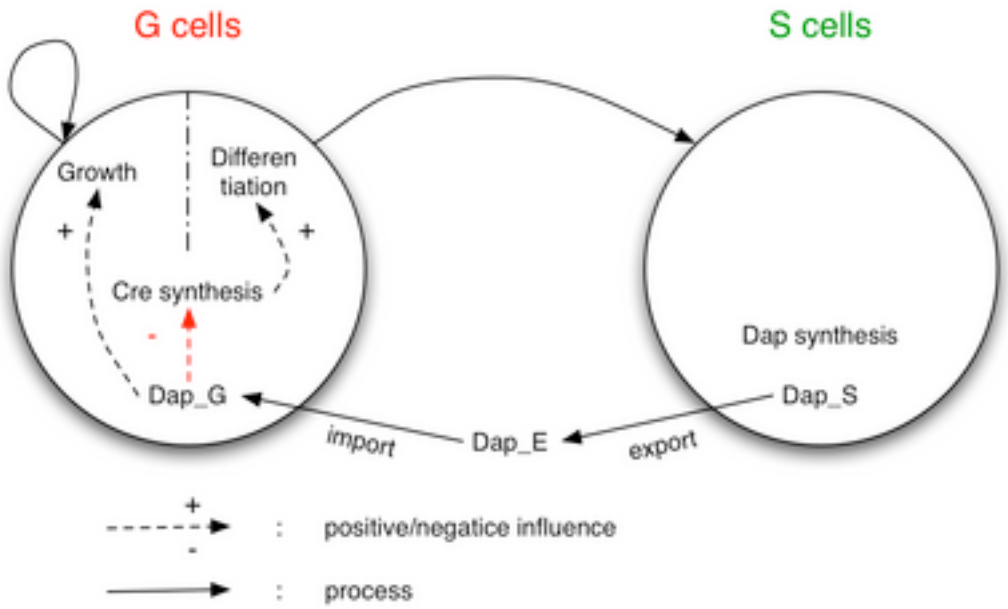
## Extended Algorithm

The extension relies on the fact that compartments elements are uniformly organized, meaning the SSA can be used in each compartment. Intuitively, at each iteration of the new algorithm, the standard SSA is evaluated on each compartment of the system ; the compartment with the smallest reaction time is updated (with respect to the corresponding reaction) and the other compartments remain unchanged. This procedure is naturally recursive and can be expressed as follows:

```
ExtSSA (S,t,R0,...,RN) = {
  (S',t') := SSA (S,t,R0,...,RN)
  foreach compartment M of S do
     let (M',tM) = ExtSSA (M,t,R0,...,RN) in
       if tM < t'
       then
         S' := replace M by M' in S
         t' := tM
  done
  return  (S',t')
}
```

This algorithm is taken from here.

**SMB in Terms of Compartments and Chemical Reactions**




We start from the same representation of the system as presented in the ODE based simulation. Obviously, bacteria are represented by compartments that contain molecules as shown on the picture of the previous section. Let now consider the figure next to this text in order to sum up the different molecules we have to deal with:

- DAP: it is of course one of the major components of the system. It can be located in the bacteria or out of them in the environment.

- Cre: it is the second main molecule to be considered. It remains in cells and cannot be exported or imported.

- DNA: we focus only on the two parts of the DNA we are interested in:

- Both types of cell are able to produce Cre. This encoding region is under the control of a constitutive promoter. We then consider a molecule DAPAp that is an abstract representation of this part of the DNA. Thus every cell contains a DAPAp molecule. Following the two possible behaviors we consider, DAPAp can be inhibited or not by DAP (here is controlled the DAP (in)dependence of the differentiation). If the case occurs, the inhibited form of DAPAp is named DAPAp_i.

- The germ cells differentiate using a Cre controlled recombination of a DNA part. This part have two different states as the considered cell is either differentiated (somatic cell) or not (germ cells). See here for a biological description of this mechanism. We can call these states LOX_Box for germ cells (the DNA part uses a LOX-Cre mechanism to recombine) and DAP_Box for somatic cells (after recombination the DNA is able to produce DAP). Once again each cell has to contain one molecule of DAP_Box or one molecule of LOX_Box.

The other components are of course bacteria compartments. The two kinds of compartment differ only by the presence of either LOX_Box for germ cells or DNA_Box for soma cells. Using these molecules, we are able to model the dynamics by chemical reactions.

DAP and Cre degradation:

$$Cre \xrightarrow{K\_CreD} .$$
$$DAP \xrightarrow{K\_DAPD} .$$

DAPAp promoter activity for DAP dependence differentiation: these rules prevent the production of Cre when DAP inhibits DAPAp

$$DAPAp + DAP \xrightarrow{K\_DAPApI} DAPAp\_i$$
$$DAPAp\_i \xrightarrow{K\_DAPApA} DAPAp + DAP$$
$$DAPAp \xrightarrow{K\_Cre} DAPAp + Cre$$

DAPAp promoter activity for independent differentiation: DAPAp is never inhibited

$$DAPAp \xrightarrow{K\_Cre} DAPAp + Cre$$

Differentiation: note that the differentiation is irreversible. Molecules of Cre have to bind the both LOX sites of the LOX_BOX. We name $LOXP\_Box_{Cre}$ the intermediary state

$$LOXP\_Box + Cre \xrightarrow{K\_Diff} LOXP\_Box_{Cre}$$
$$LOXP\_Box_{Cre} + Cre \xrightarrow{K\_Diff} DAP\_Box$$

DAP production: this only happens in somatic cells, germ cell does not contain DAP_Box. Therefore, only somatic cells are to feed germ cells

$$DAP\_Box \xrightarrow{K\_DAPiP} DAP\_Box + DAP$$

DAP import and export: these rules are applied in the environment. The square brackets represent compartments (for example $[...]_{BactS}$ is a somatic cell)

$$[DAP, ...]_{Bact} \xrightarrow{K\_DAPEx} DAP + [...]_{Bact}$$
$$DAP + [...]_{Bact} \xrightarrow{K\_DAPIm} [DAP, ...]_{Bact}$$

Bacteria macroscopic evolutions (division and death): when a bacterium dies, its internal DAP molecules are released in the environment. As DAP is essential for germ cells division, we can envisage that the value of the kinetic constant $K\_Div$ is a function of the number $n$ of DAP molecules in the cell

$$[n.DAP, ...]_{BactG} \xrightarrow{K\_Div} [(n/2).DAP, ...]sub>BactG</sub> + [(n/2).DAP, ...]sub>BactG</sub>$$
$$[n.DAP, ...]_{Bact} \xrightarrow{K\_Death} n.DAP$$

**MGS Implementation** 


As presented here, the MGS language provides a specific rules application strategy implementing the SSA. We propose to implement the algorithm `ExtSSA` using MGS. The program (available here) is briefly described with the representation of the system, the encoding of chemical reactions and a proposition of optimization for `ExtSSA`.

### *State and Structure*

First of all, our system handle different types of molecules. They are represented in MGS by symbols (back quoted strings):

- `` `DAP ``: DAP molecules
- `` `Cre ``: Cre molecules
- `` `DAPAp ``: DAP sensitive promoter (inhibited form: `` `DAPAp_i ``)
- `` `LOXP_Box ``: DNA configuration of germ cells (Cre bounding form: `` `LOXP_Box_Cre ``)
- `` `DAP_Box ``: DNA configuration of somatic cells

As mentioned above, the system is represented by nested compartments. MGS provides the so-called *bag*, a particular data structure corresponding to multi-sets (sets where multiple occurrences of the same element are allowed) whose topology is a complete graph. In other words, each element of the bag is neighbor with all the others. From the dynamical systems point of view, the neighborhood is equivalent to a potential interaction: in bags, each element can react with all the others. This topology is then perfect to represent well-mixed chemical solutions and, of course, compartments. The system is composed of 3 types of compartments defined here:

- the environment of type `Env` where all bacteria are localized
- the germ cells of type `BactG` that must contain one molecule `` `LOXP_Box ``
- the somatic cells of type `BactS` that must contain one molecule `` `DAP_Box ``

The value `` (`DAP::`DAP_Box::BactS:())::(`LOXP_Box::BactG:())::`DAP::Env:() `` is an example of a system state. It is composed of two bacteria, a germ cell and a somatic cell (the somatic contains one molecule of DAP), and an outside diffusing DAP molecule.

### *Dynamics*

The chemical reactions are specified using transformation rules (see here). There are 14 different reactions embedding in two different transformations:

- transformation `inBact`: this transformation specifies the chemical reactions occurring in bacteria (independently if they are germ or somatic). The reactions

are chemicals degradation, DAPAp promoter inhibition, differentiation and DAP production (for somatic cells only).

- transformation `inEnv`: this transformation specifies the chemical reactions occurring in the environment. Reactions are DAP degradation, DAP import and export (this rule actually appears in `inBact` to improve the efficiency of the program and to noticeably reduce the execution time).

As an example, the following rule appears in `inBact`:

```
`DAPAp, `DAP ={ C = sndOrder(K_DAPApI,1.0) }=> `DAPAp_i ;
```

It specifies the inhibition of the promoter DAPAp by a molecule of DAP: `` `DAPAp `` and `` `DAP `` reacts to produce `` `DAPAp_i ``, the inhibited form of `` `DAPAp ``. In the arrow, the parameter `C` defines the stoachstic constant of this inhibition. It is computed using the function `sndOrder` that takes as arguments

- `K_DAPApI`, the kinetic constant of this reaction (whose value is defined here), and
- the volume of the cell (we assume here that the volume is always 1.0 ; volume dependence is not implemented yet).

## *Optimization*

The previously described `ExtSSA` is not straightly implemented. In fact, while stochastic simulations can deal with a small number of molecules, they often exhibit limitations as they are time greedy. On the first hand, events are triggered asynchronously: only one reaction is applied at each time step. As a consequence, a simulation requires a lot of time steps to be significant. On the other hand, choosing the next reaction requires to draw a lot of random numbers. Unfortunately, this operation is badly time consuming and increases the execution time.

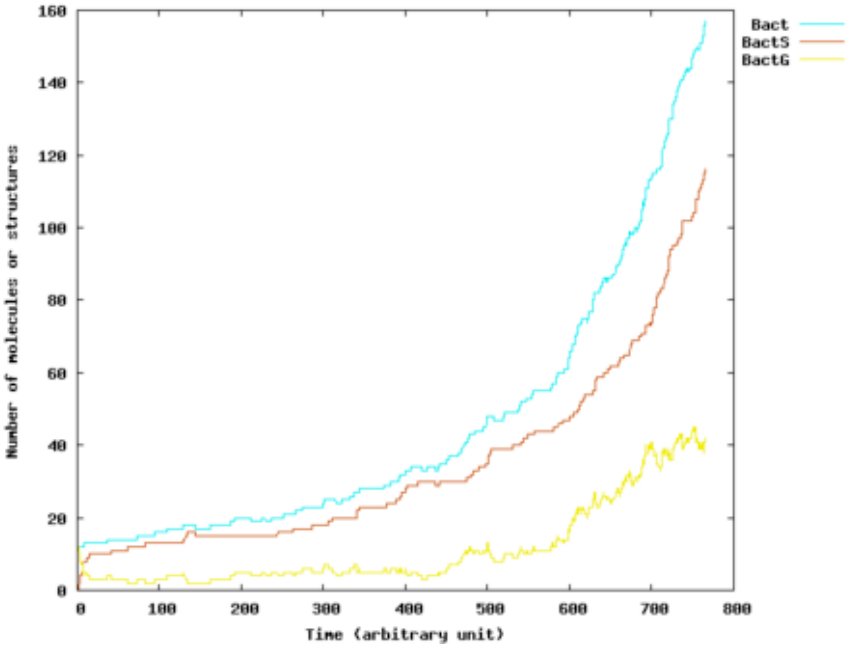
We propose to decrease the number of random numbers generations by freezing cells that do not evolve. This improvement consists in using a scheduler that stocks cells with their reaction time in a buffer (the program is available at here). At each time step, the SSA is evaluated on the environment. If the returning reaction time is smaller than the smallest reaction time of the buffer, then we make the environment evolves and reaction times of cells touched by this update are recomputed and the buffer updated. On the other, if the environment evolution is slower, the fastest reaction of the buffer is applied and the corresponding cell is updated. This improved version of `ExtSSA` is programmed here.

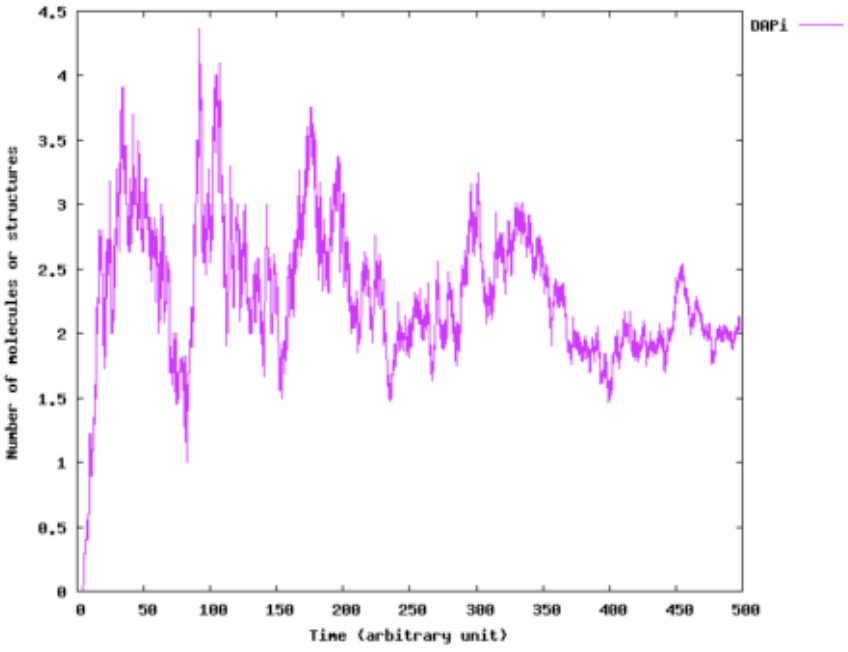
To give an idea of the efficiency of our improved algorithm, the evaluation of a 100000 steps evolution starting with a population of 20 cells to reach a population size of about 230, is about 290s on a standard computer. Moreover, note that the MGS top-

level is a prototyped and generic system that clearly not provides the efficiency of an *ad-hoc* programming of our improved `ExtSSA`.

## Some Results

The following figures was generated by the implementation of `ExtSSA` we have just described. We start with 20 germ cells without external DAP production. On the left, the temporal evolution of the bacteria population size. The exponential growth of the population is once again stressed by the simulation. On the left, the graph presents the mean number of DAP molecules in a cell. As we can see, this never goes above 5 molecules. It justifies the use of a stochastic process for realistic simulations.



### Conclusion of stochastic simulation

We have developed a complex and generic framework for stochastic simulations of nested membranes systems following ideas presented here. Moreover, our implementation provides an optimization that allows to use this framework in a life-size project.

On the other hand, the simulation is not volume-dependent. As we do not take into account the evolution of the cells size, reactions rates are not yet well computed. This is a work in progress. Our simulation is also limited because of the homogeneous assumption of `ExtSSA` in compartments: the somatic cells feed all the germ cells. But as previous simulations show, somatic cells only feed germ cells that surround them. The spatial distribution of cells is not here taken into account. A possible extension is to merge the three simulations to deal with all the aspects of the system:

- the cellular automaton to simulate DAP diffusion in the environment
- the spatial model to simulate the spatial organization of cells
- the **Gillespie based simulation** to simulate the chemical reactions occurring in cells

## Summary of modelling

The goal of our modeling work was to test our design, mainly to identify potential flaws of the system at early developmental stages.

In part 2, we showed that the system can present – at least qualitatively – the desired behaviour: an exponential growth of the two populations of coexisting cellular types.

In part 3, our results indicated that the system's behavior should be reasonably robust, and provided arguments in favour of the design having a negative regulation of recombinase expression by Dap (increased robustness and tunability).

In all cases, models incorporating additional details, related to space and/or stochasticity, indicated that these phenomena should not affect the global behavior of the system. So previous conclusions, obtained using deterministic models, should remain valid despite the fact that we neglected noise- and space-related issues. This result is not surprising. Intuitively, even if SMB system is heterogeneous (because it is composed of two distinct populations of cells), the spatial distributions of both cells types are uniform; thus, the system evolution is space independent. Nevertheless, we were interested in checking the property using models and simulations. In fact this led us to use and develop original techniques (like Delaunay triangulation or extended to nested membranes systems Gillespie algorithm). From a general point of view, developing such techniques is also of great interest for synthetic biology. Following the concepts of decoupling and abstraction that characterize biosynthetic developments, we have to ideally validate and study designs before constructing them physically. These

validations appear at each step of a standard development, at the levels of systems, devices and bricks designing. We tried to follow and contribute to this methodology by considering at first phenomenological and global models, and molecules scaled models at last.

Getting back to SMB, we would like to stress here that these results should be taken with great care, given the extreme simplicity of our models and the lack of data to provide information on parameter values and initial conditions. But still, globally...
**...all these results corroborate our initial design**.

## Appendix of modeling
### Tools Description

For our simulations we used unusual tools, Biocham and MGS. Thanks to their specificities and capacities, we were able to simulate easily the mechanisms that we wanted to focus on.

## Biocham

BIOCHAM is a programming environment for modeling biochemical systems, making simulations and querying the model in temporal logic.

**http://contraintes.inria.fr/BIOCHAM/**

## MGS

MGS is an experimental programming language developed at the university of Evry and dedicated to the modeling and the simulation of dynamical systems with a dynamical structure. We briefly present in this section the philosophy of MGS programming.

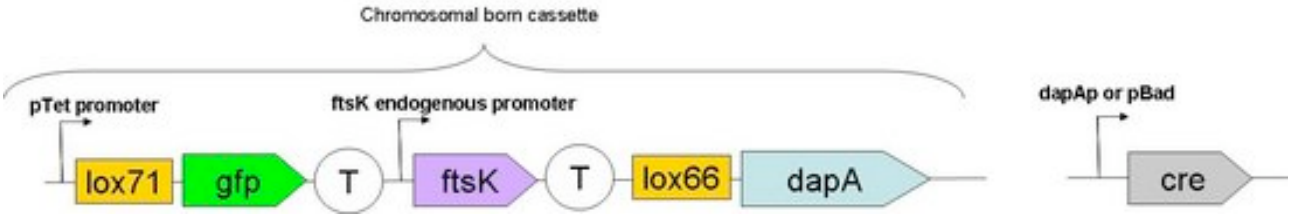
## Sources of MGS and Biocham programs


http://parts.mit.edu/igem07/index.php/Paris/Sources

# 4. Construction Process From IGEM07

## Overview

Through classical molecular biology techniques, we aimed to construct the following genomically inserted cassette

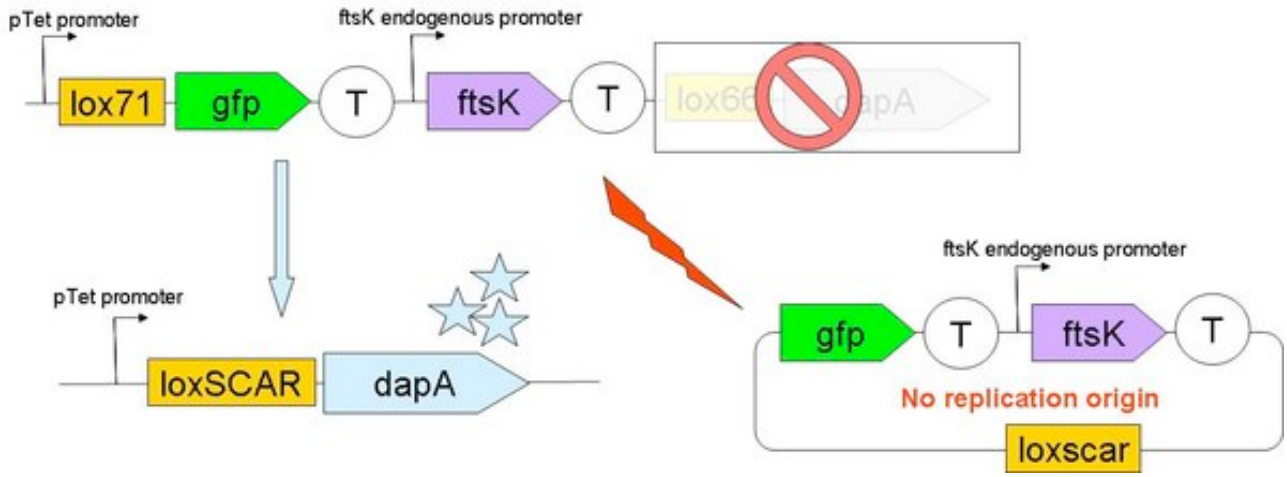



This full construct has yet to be assembled in the genome.

As can be seen on this schematic representation of the SMB genomic backbone cassette, at basal genomic state (in Germ line cells):

- pTet promoter drives the expression of gfp.
- The expression of ftsK is controlled by its natural promoter.
- ftsK is isolated from pTet promoter by the intercalation of Terminator (B0015 terminator).
- dapA gene is in a dormant state since it lacks a promoter to drive its transcription.


Upon G to S differentiation, the following genomic reassembly should take place: constructed. These are termed the "**upstream construct**" & the "**downstream construct**":



Cre mediated lox recombination should lead to:

- excision of ftsK gene from the genome on a circular unreplicative DNA molecule. We ignore how much time such a DNA is stable in a cell.
- placing dapA gene under the control of pTet promoter. This should lead to dapA expression, & hopefully to DAP synthesis.

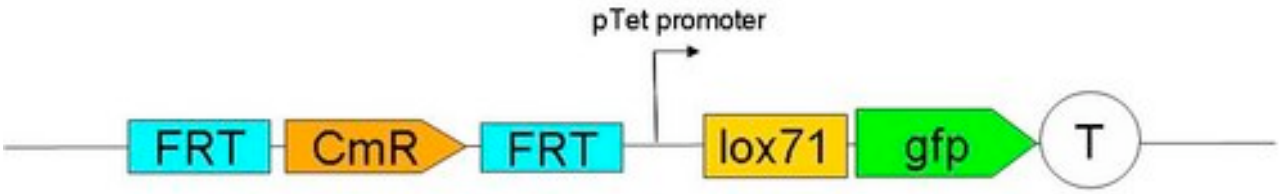
In order to construct this genomic cassette, we have considered two technical solutions:
1) Either the full construct is cloned on a plasmid and inserted in the genome of a *dapA*-, *ftsK*-(ts) E.coli strain. This was the initial strategy we adopted then subsequently abandoned. cloning ftsZ gene, which we had initially selected as the essential gene in our construct, has proven to be quite problematic. 2) Or the cassette is sequentially assembled, in the genome, around the essential gene selected (ftsK in our case).

The second strategy does not require cloning ftsK gene & does not require using an *ftsK* mutant.

We have chosen the second strategy mentioned above to generate the SMB genomic backbone construct. The different steps of this process are as follow:

2 plasmid born constructs were generated. These are termed the “upstream construct” & the “downstream construct”:

**Upstream construct:**




SEE HERE how the upstream construct sequentially assembled on a plasmid.

**Downstream construct:**

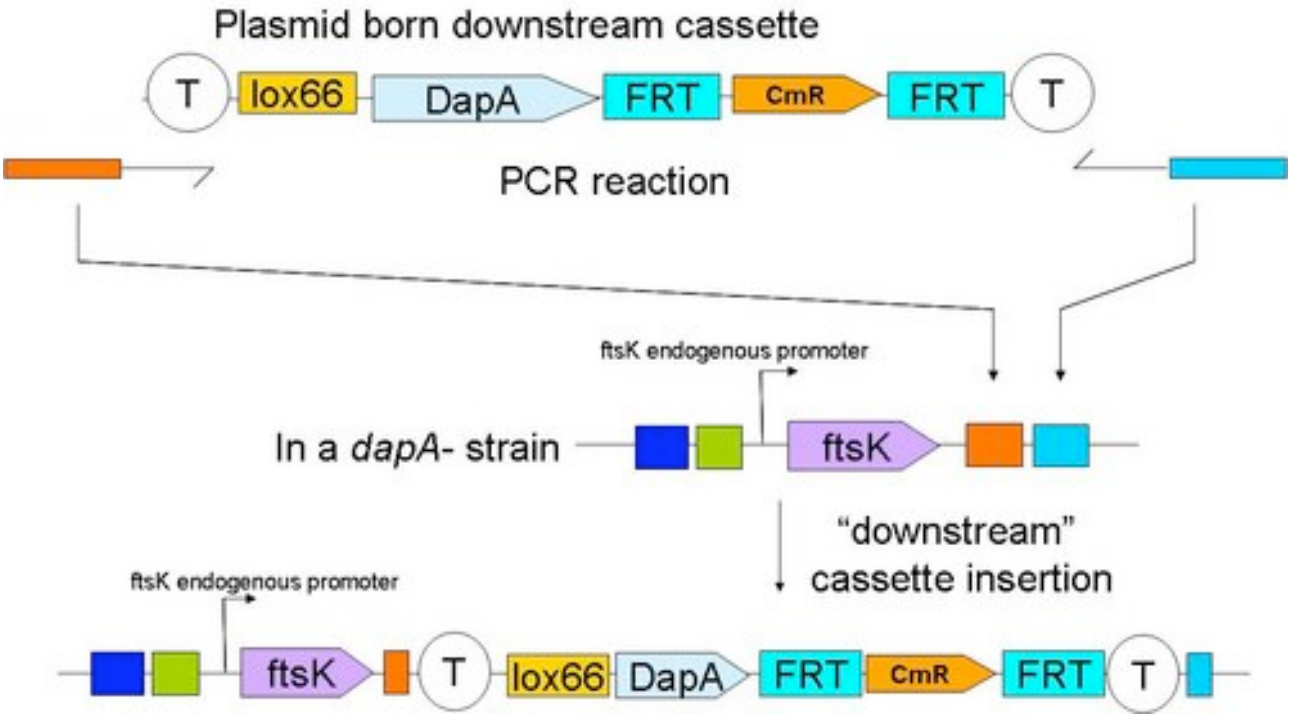

SEE HERE how the downstream construct was sequentially assembled

As their names indicate, these constructs are to be inserted upstream and downstream of ftsK gene in the genome of a *dapA*- strain.

We chose to insert these constructs using a strategy described by Datsenko KA. & Wanner BL. (PNAS 2000)

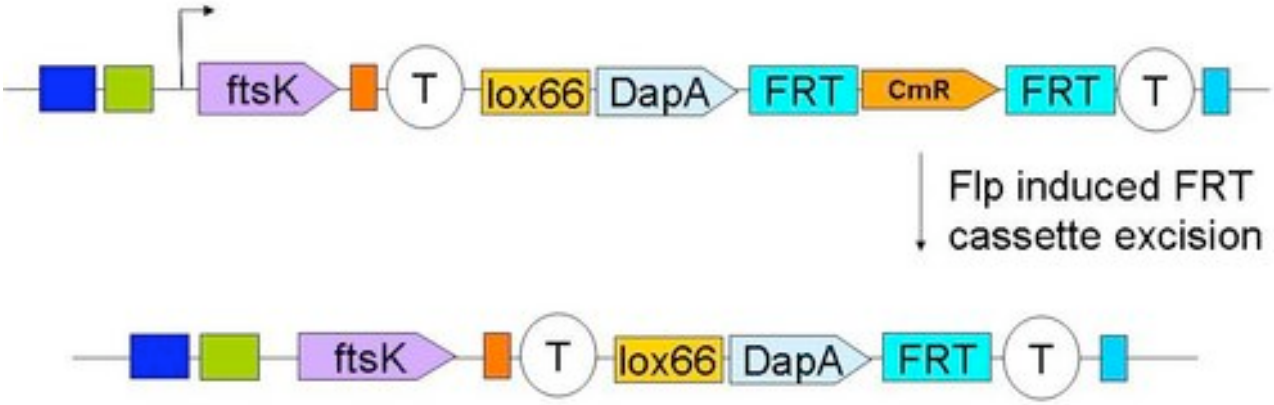
We have yet to insert the constructs in the genome. This will be done sequentially:

In a first step the downstream construct is amplified using oligonucteotides baring sequences homologous to those downstream of ftsK. The PCR product is used to transform a dapA- E.coli strain.

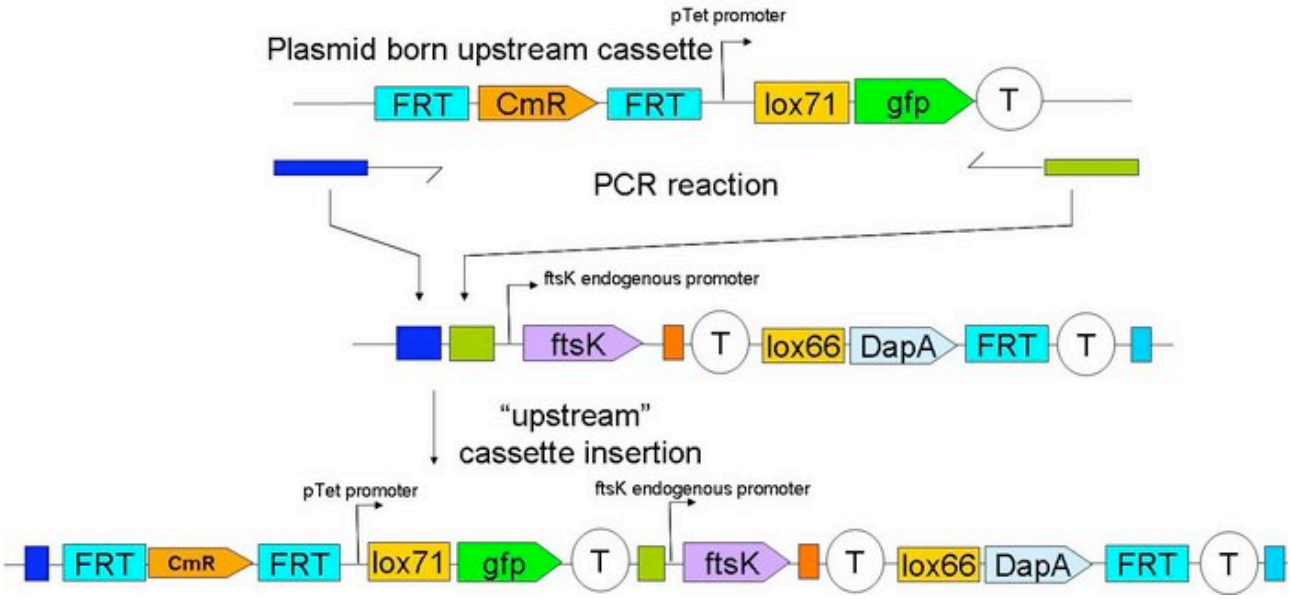



The resistance cassette frt-CmR-frt carried by the downstream construct allows screening for the event of genomic insertion of the construct. CmR gene confers resistance to the antibiotic chloramphenicol.

Subsequently, the Chloramphenicol resistance gene is eliminated by FRT recombination. This reaction is to be catalysed by the site specific recombinase: Flp.




The upstream construct can then be inserted based on the same strategy.



# 5. Paris/Results From IGEM07

**Contents**

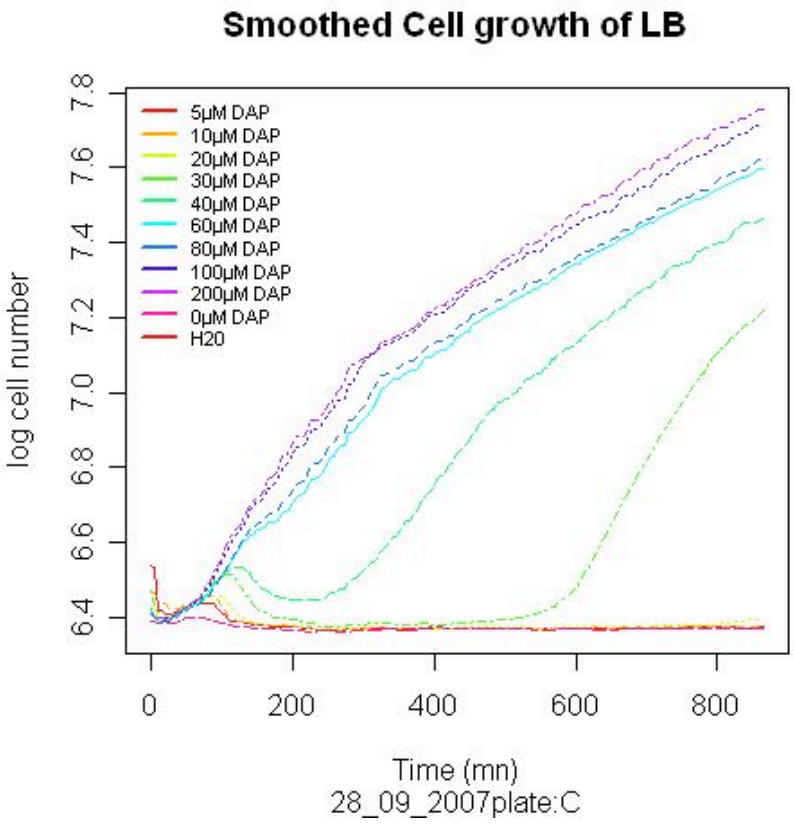


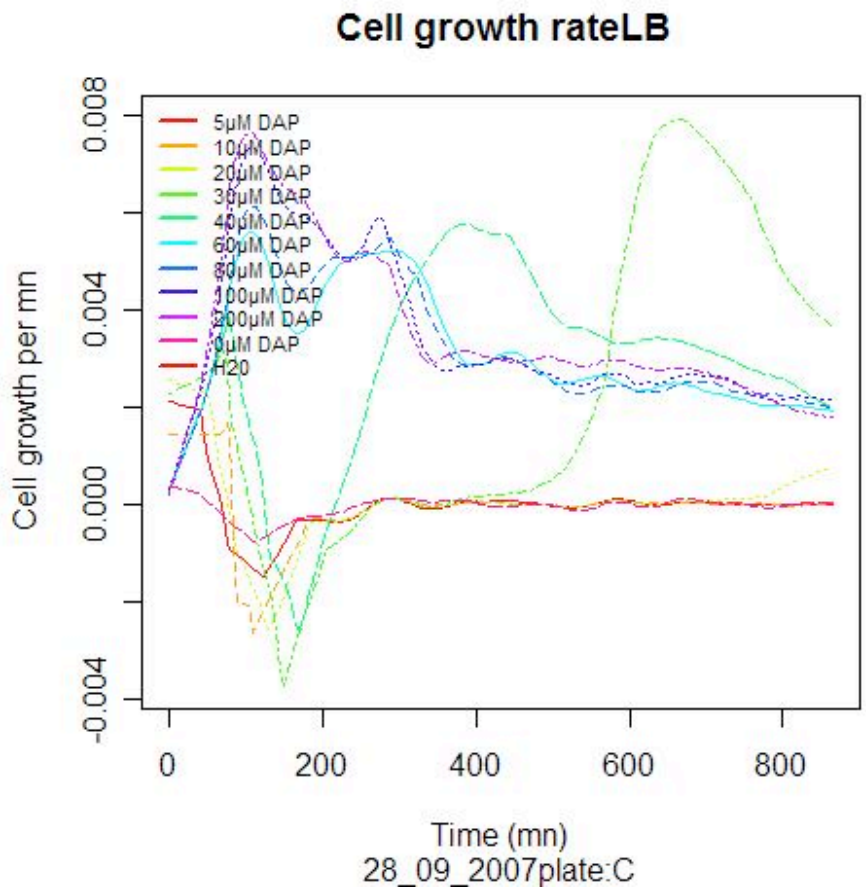
## 5.1 Experimental results

### *5.1.1.1SMB related experiments*

**dapA deletant growth**

We expect the somatic cells to excrete only a limited amount of DAP, it would thus be nice if our auxotroph strain could grow on limited amount of metabolite. Over night culture were diluted 100 time in LB suplemented with different amount of DAP.






The experiment describbed above has been performed on 28/09/07

Several such experimens were performed at other dates
06/08/07

28/09/07

29/09/07

A correspondance between optical density and number of cell was used to plot the real cell growth curves, as well as the growth rate ( dX/dt / X). These curves allowed after smoothing, to extract parameter for the models (max growth rate, death rate..)

### *5.1.1.2 Survival of dapA deleted strain and Co-culture experiments*

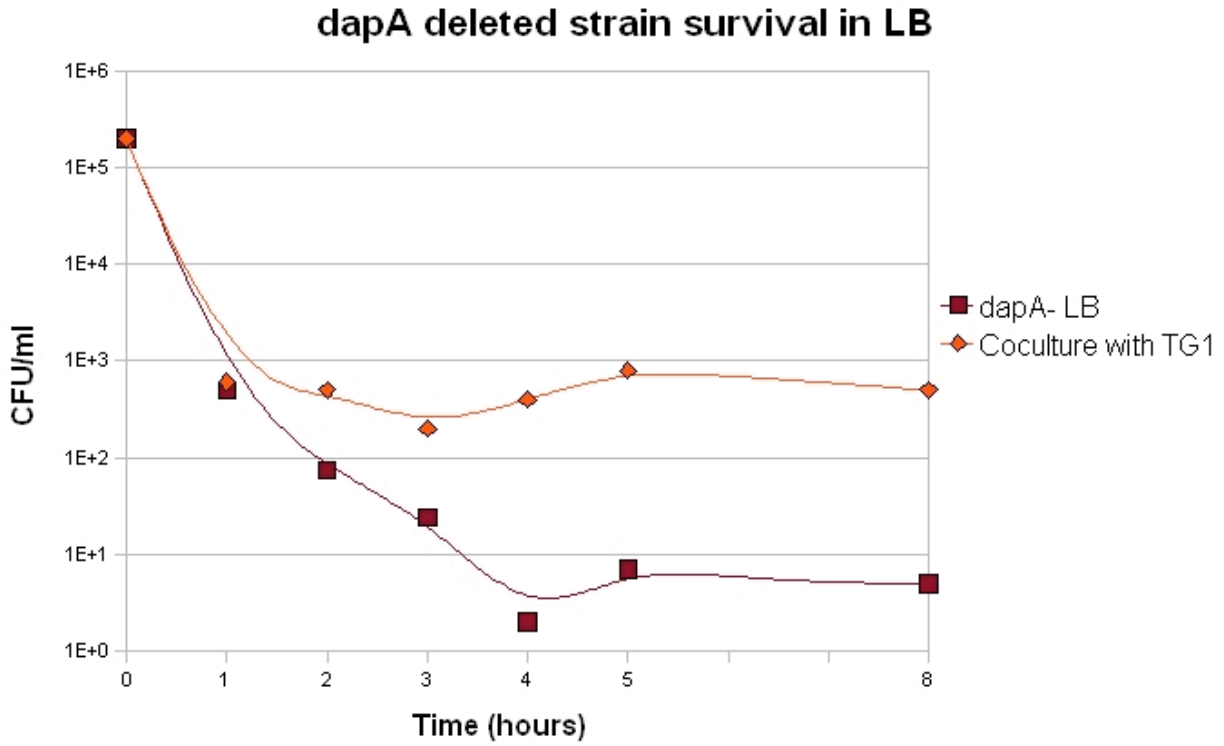
If we want our system to be robust, the germ line cells should be able to live as long as possible when deprived of DAP. They may indeed face such a feat within our system if their is not enough somatic cells at a given time.

Our dapA deleted strain was grown over night in LB + 300$\mu$M DAP. A culture was then launched diluting 100 time the ON culture in LB (+ Kanamycin). 100$\mu$L were plated each hour and the number of clone determined.

We also want to know if the soma of our synthetic organism will be able to feed the germ line. Unfortunatly, this cannot properly be done without the final construct. Nevertheless, we can already check if in similar situations, dapA deleted strain can survive.

Two different co-culture experiments were performed:

- In the first one, the dapA deleted strain is grown with a prototroph strain. In this case, we know for sure that the prototroph cells will take the population over. Nervertheless it is interesting to see if dapA deleted strain survive longer in this condition than if alone and without DAP.




(the experiment has only been done once, and would be worth repeating to confirm the results)

We can draw from this curves two important conclusions:

1. Most of the cells die within the first 2 hours of culture, but a small fraction of the cells are still alive after 8 hours. A part of the dapA deleted cells can thus survive for quite long, which is a good point for us.

2. When in coculture with a prototroph cell, around 100 time more cells have a long term survival. This means that the dapA deleted strain benefits a bit from the prototroph cell presence and its DAP production capacities.

- In the second coculture experiment, the dapA deleted strain is grown with a strain bearing an auxotrophy to another metabolite. This experiment reproduces cross-feeding works done on the yeast by Shou W et al. (Synthetic cooperation in engineered yeast populations, PNAS). In this coculture there is a mutual dependence of each strain for the other. If this works, then we know for sure that dapA deleted strain can be rescued by a strain producing DAP!

Our dapA- strain was grown with a tryptophan operon deleted strain. The culture was plated to check the presence of both strains after 8H. They were both present in concentration above 5*10^4 CFU/Ml. This clearly means that DAP is a good choice as our auxotrophy metabolite !

### *5.1.1.3 DAP excretion by prototroph cells*

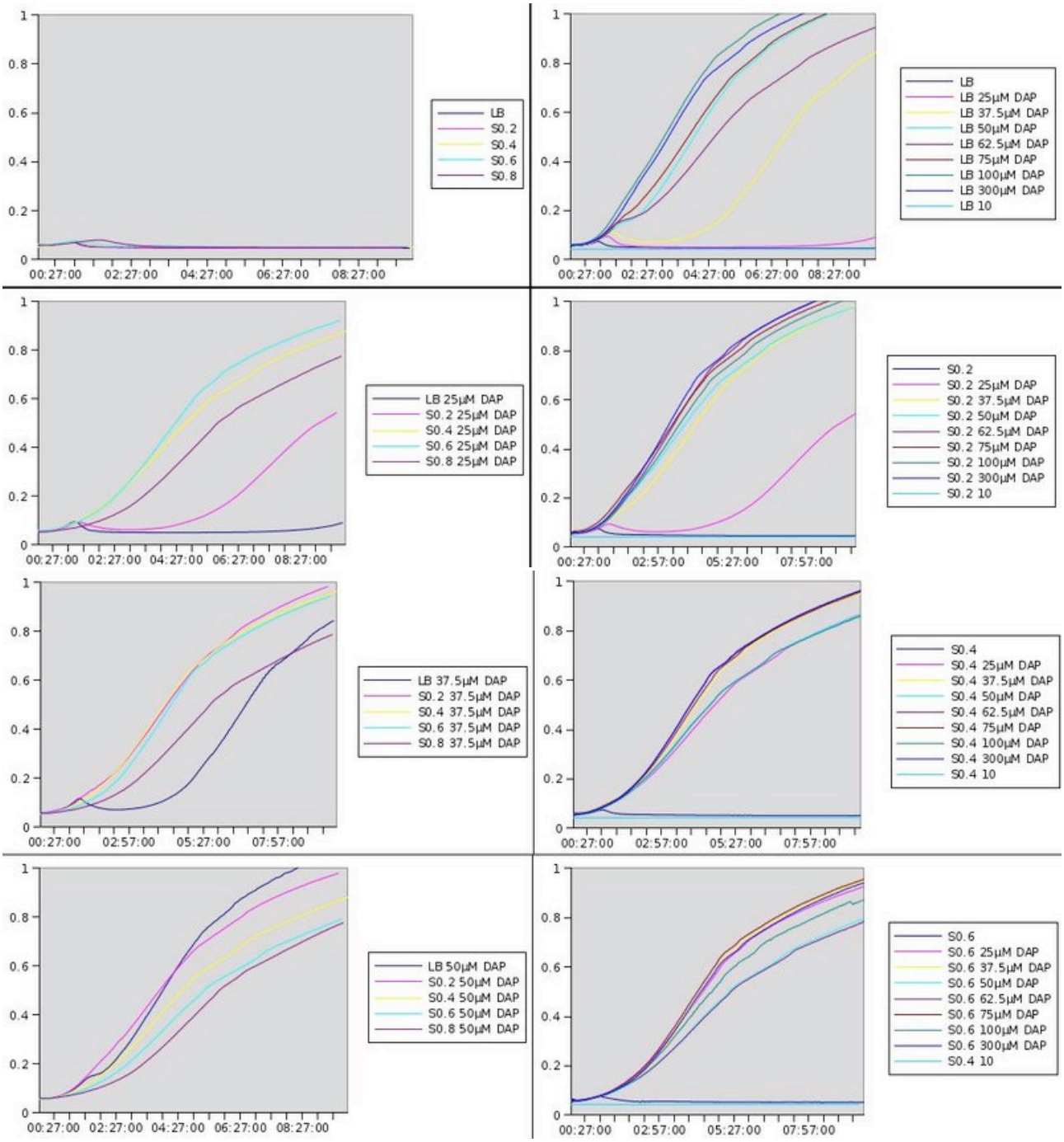
We want to know if prototroph cells are excreting DAP and in what amount. Instead of doing proper chemical dosages which are expensive, we had the idea to make a biological measurements. If we want to quantify DAP concentration of a given medium, we might be able to do so simply by looking at how well a dapA- strain grows into it.

Prototroph MG1655 strain was grown in LB, and the medium was recovered at different stages of the culture and purified. The dapA deleted strain was then grown in the recovered medium. What we observed is that no enough DAP is present for any growth to happen. From this point we decided to add little concentration of DAP into the recovered medium, to see what is the minimum amount to add to obtain growth. If this amount is small then it means that there is already quite a lot of DAP present into the medium. Typically if we need to add 10$\mu$M of DAP to get a growth similar to LB supplemented with 20$\mu$M, we can estimate that the initial concentration is around 10$\mu$M.

More rigorously, what we measure isn't really the DAP concentration of the medium, but rather what we could call DAP equivalents. This is mainly due to two points. First, dapA gene is the first out of 5 genes making the steps from aspartate-semialdehyde to DAP. So we measure in fact the sum of all intermediaries of the pathway that may be present in the medium and imported by the cells. This is not a problem for us since we do not really care with what exact compound the soma feeds the germline. Second, the recovered medium isn't really LB anymore, so it is not perfectly rigorous to compare it with LB. There might be other compounds excreted during growth, compensating for the DAP starvation. But we do not really care either if this is the case. What we really want to see is how much the excretions of a prototroph cell can favor the growth of a dapA- strain (regardless of what excreted compound matters). And we measure this as DAP equivalents.

Here are representative results of what we got from this experiments:

Medium recovered from MG1655 culture broth are annotated S0.2, S0.4, S0.6, S0.8 and were recovered at optical densities of respectively 0.2, 0.4, 0.6 and 0.8. The added DAP concentration is given.




We clearly see that the latter we recover the medium in the prototroph growth, the less DAP we need to add to obtain growth. This is one more hint that DAP is a good choice for the SMB and we can also try to estimate DAP equivalents for our media. For instance the growth in S0.2 with 25$\mu$M of added DAP seems equivalent to the growth in LB with 37.5$\mu$M added DAP. This means that S0.2 contains around 12.5$\mu$M of DAP equivalents. Nevertheless it was quite hard to retrieve any reliable data from this experiment.

By using the experiment of the 06/08 and of the 28/09 we find the production of DAP by 2 different strains to be :

| | |
|---|---|
| S0.2 | 10 µM |
| S0.4 | 20 µM |
| S0.6 | 25 µM |
| S0.8 | 28 |

The experiments described above have been performed at various times

28/09/07

http://parts.mit.edu/igem07/index.php/Paris/September_28

29/09/07

http://parts.mit.edu/igem07/index.php/Paris/September_29

### *5.1.1.4 Recombination frequency measurements*

In the section “Design Process”, the question of the recombination frequency has been addressed. G to S differentiation frequency, and thus lox recombination frequency on which it is based, is a key aspect of our system. Indeed, optimal overall differentiation frequency lies somewhere between 0 and 50% per generation.

We would like to have tunable recombination device in order to find the optimal frequency, which lies somewhere between 0 and 50% of recombination per generation. We should be able to tune the recombination frequency by modulating the Cre recombinase expression. In order to do this, we have cloned Cre under the control of the pBAD promoter. We then wanted to characterize our "Cre generator device", and determine the relation between Cre expression level and recombination frequency.

Three types of experiments were planned and partially performed in this regard:

- Experiments using FX85 strain harboring lox-KmR-lox cassette: indirectly measuring recombination frequency by plating on Kan plates after Cre expression assays.

- Experiments using FX85 strain harboring lox-KmR-lox cassette: indirectly measuring recombination frequency by studying growth rates.

- Experiments using the recombination measurement biodevice.

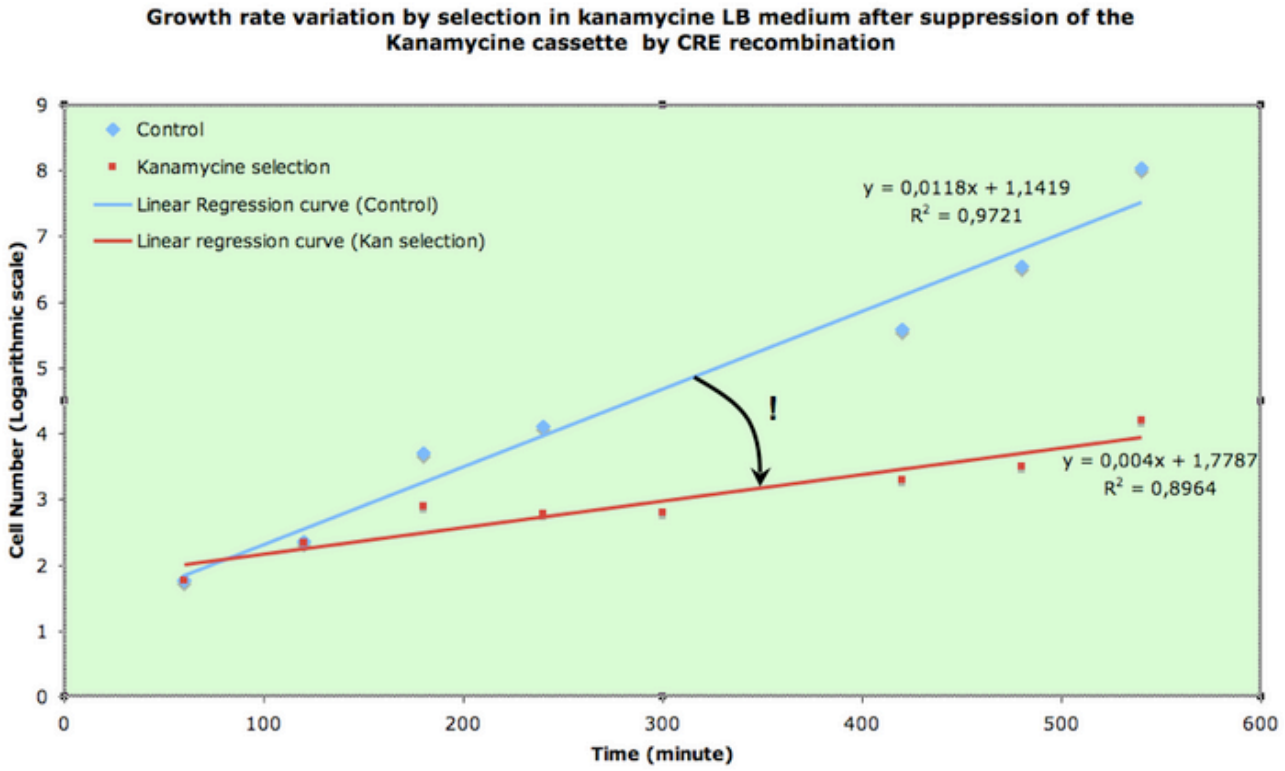
A) *Experiments using FX85 strain harboring lox-KmR-lox cassette: indirectly measuring*

We used FX85 strain (provided by Francois-Xavier Barre) carrying the cassette: lox-KmR-lox inserted into its chromosome. We transformed this strain with our pBad-Cre construct (carried by an Ampiciline resistance plasmid: pSB1A2). Inducing of Cre expression with different arabinose levels was performed. The last step is then spreading on selective plates (Amp or Amp+Kan). The ratio between the number of clones on the two types of plates should give us an estimate of the recombination frequency. When we tried to do this, we did not get any clones on the Amp+Kan plates, even without Cre induction. But this doesn't mean that the recombination frequency is 100%. In fact, if the recombination frequency is around 50% per generation or higher, no colonies can grow on kan plates, simply because half or more of the cells will die at each generation (those who recombine are not resistant to Kanamycin any more after little time).

This means that our Cre generator is quite leaky and gives already quite high recombination rates without induction.

*B) Experiments using FX85 strain harboring lox-KmR-lox cassette: indirectly measuring recombination frequency by studying growth rates.*

If recombination frequency is two high in our experimental system, then no growth can be seen on Ampiciline (plasmid selection antibiotic)+ Kanamycin (screens against recombinants: cells having excised lox-kanR-lox from their genome). We tested an alternative strategy in order to circumvent this problem. In order to determine what recombination rate we had, we performed another experiment. FX85 strain was transformed with our pBad-Cre construct. Directly after transformation, liquid cultures were launched with either Amp or Amp+Kan. 100µl of the cultures were regularly plated on LB+Amp, giving the following growth curves:



The number of cells in the culture selected with ampiciline is given by:

$$Namp = Namp_0 * 2^{(t/T)}$$

where T is the generation time and t the time.

The directing coefficient of the log regression curve is thus:

$$y = \log\frac{(2)}{T} = 0.0118$$

which gives:

$$T = \log(2)/0.0118 = 25.5\,min$$

We thus have a generation time of approximatively 25min which is
the standard value in rich medium
The number of cells in the culture selected with ampiciline and kanamycine is given by:

$$Nkan = Nkan_0 * [2(1-r)]^{(\frac{t}{T})}$$

where r is the recombination rate.
The directing coefficient of the log regression curve is thus:

$$y' = \log\frac{(2(1-r))}{T} = 0.004$$

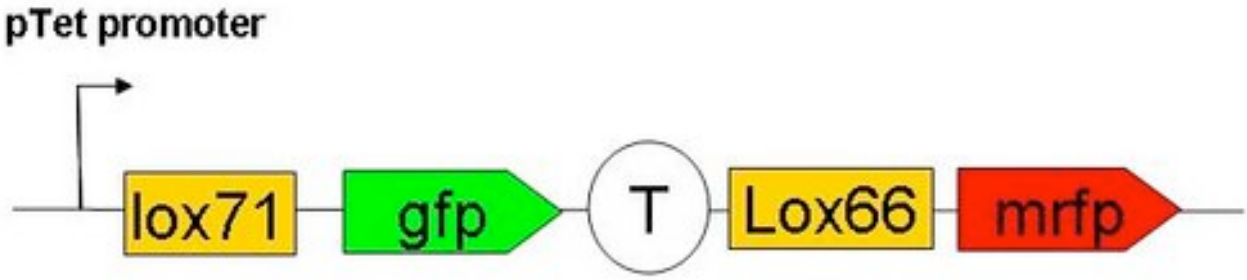
This gives:

$$r = 1 - 10^{(y'*T)}/2 = 0.368$$

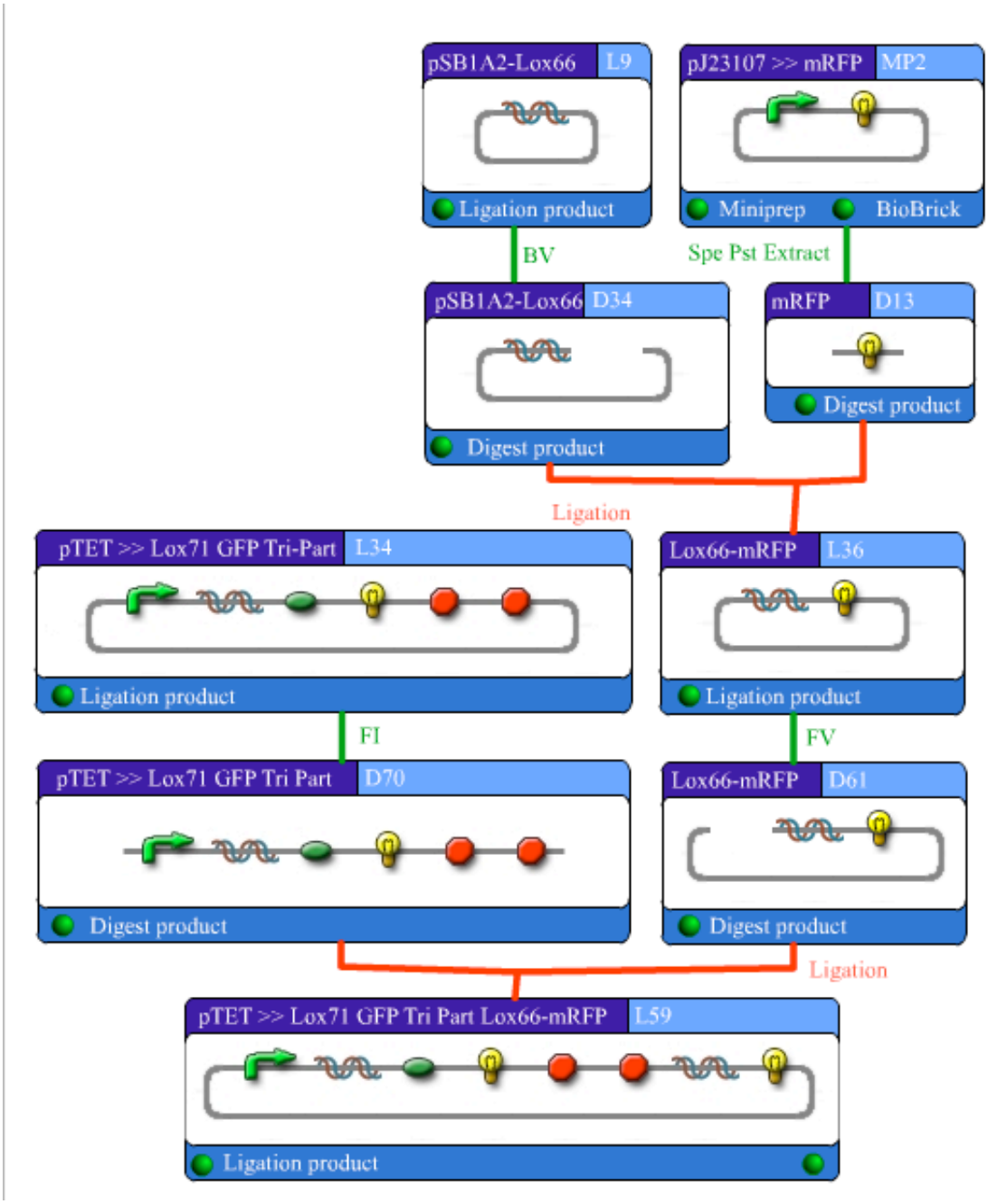
in this experiment the recombination rate is thus 36.8% per generations.

*C) The third strategy is based on a more direct observation of recombination rate.*

We have constructed a “Recombination frequency measurement” device. The schematic structure of this genetic construct is as follows:



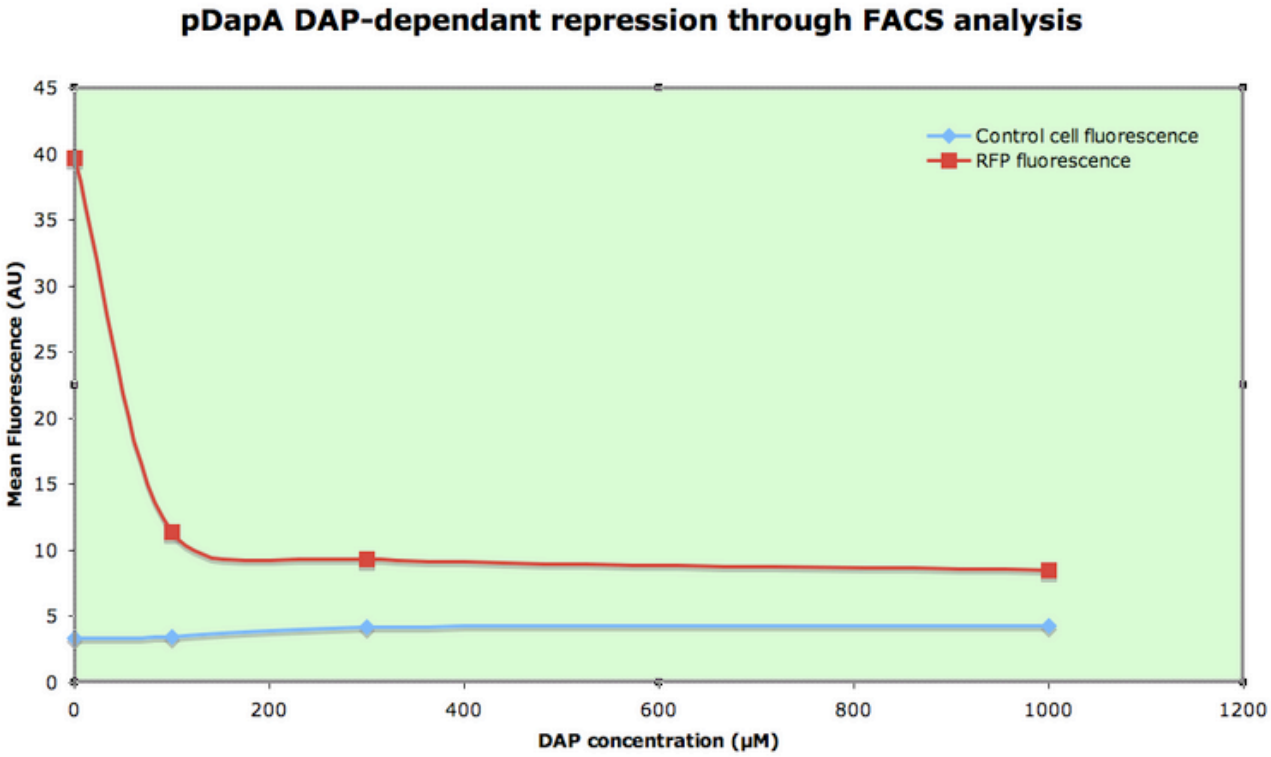
This consctruct has been generated:




Each box represents a reaction product, either digestion or ligation. Each box includes a green dot which is a link to the page of the date at which the reaction was performed, in the notebook.

This construct has yet to be inserted in the genome. Cre induced recombination frequency measurements will then be performed. Using this system, an event of recombination is accompanied by a switch in fluorescence from GFP to mRFP wich can be followed under microscope on small E.coli populations.

### *5.1.1.5 dapAp characterisation*




Knowing that cells cannot grow in a 0$\mu$M DAP environment, we then simulated it by dilluting overnight 300$\mu$M DAP LB culture at 1/100e in minimal medium M9 (in order to slow down the growth speed) without DAP during 1h before analysis.

All analyses are done on 1/100 dillution of overnight LB culture made in minimal medium M9 at 4°C.

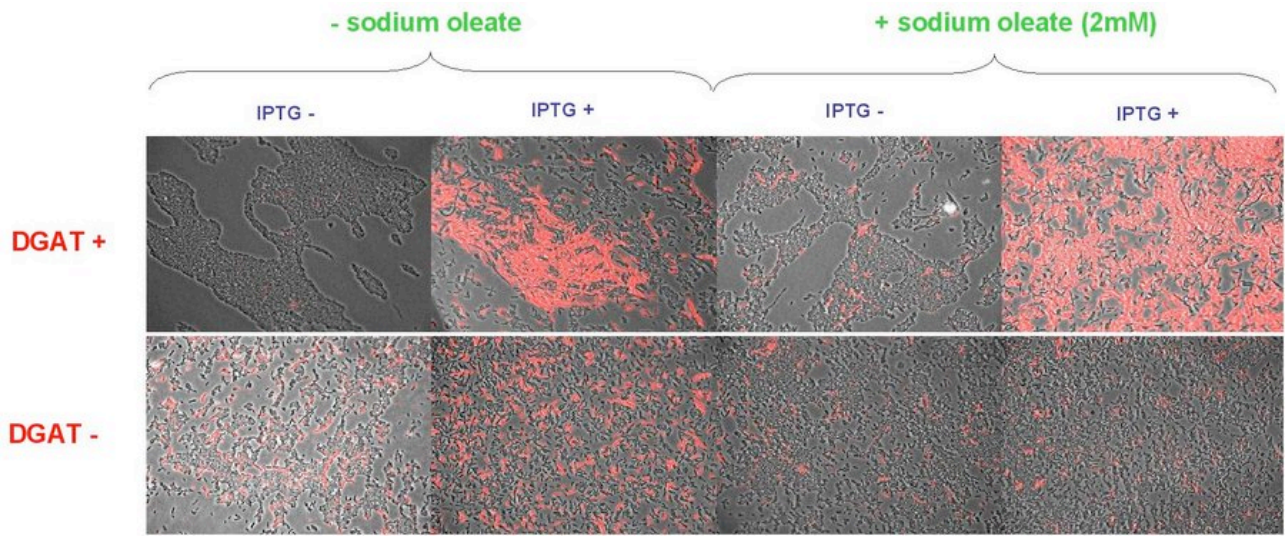
## 5.1.2 DGAT cloning and triglyceride synthesis in E. Coli

- **TG synthesis experiment**

1. We transformed chemically competent E.coli (DH5alpha) with pBluescript SK minus vector (Stratagene) (ampicilline resistance and pLac promoter) baring DGAT gene (pKS::DGAT). In this vector, dgat transcription is induced by IPTG.

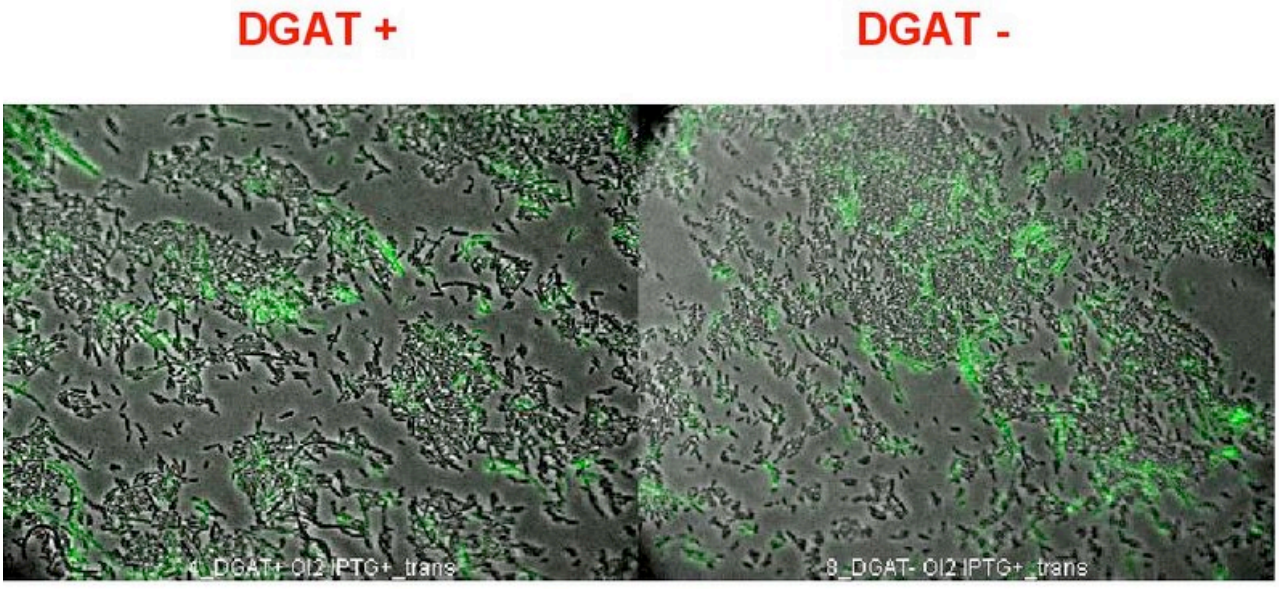
Nile Red fluorescence dye was used, at a concentration of 5$\mu$g/mL to monitor lipid inclusions in different conditions of DGAT expression and fatty acid availability:

(0.4mM IPTG induction in LB medium with or without sodium oleate 2mM). Results are shown below.




Line 1 represents E.coli transformed with pKS::DGAT; and Line 2 the negative control (E.coli transformed by part B0015). Columns 1 and 2 are LB medium without sodium oleate supplementation; columns 3 and 4 are LB with sodium oleate supplementation (2mM). Columns 1 and 3 are without IPTG; columns 3 and 4 with IPTG induction (0.4mM).
**We can observe lipid inclusion into E.coli transformed by pKS::DGAT with IPTG induction. No significant difference is seen between the +/- oleate cells.**

2. To exclude that the fluorescence observed is due to DGAT induced cell death. A cell death marker (green) is used. It can be seen below that cell death is not increased upon DGAT expression.




both with sodium oleate and IPTG

3. We started creating DGAT biobrick BBa_I718002:

- PCR based mutagenesis was performed to eliminate a PstI site in dgat coding sequence.
- We attempted adding biobrick prefix and suffix sites to dgat but have yet to finish the cloning process :-(

## 5.2 Modeling results

The goal of our modeling work was to test our design, mainly to identify potential flaws of the system at early developmental stages. An extensive description of simulation & mathematical tools is provided later.

- In part 2, we showed that the system can present – at least qualitatively – the desired behaviour: an exponential growth of the two populations of coexisting cellular types.
- In part 3, our results indicated that the system's behavior should be reasonably robust, and provided arguments in favour of the design having a negative regulation of recombinase expression by Dap (increased robustness and tunability).

In all cases, models incorporating additional details, related to space and/or stochasticity, indicated that these phenomena should not affect the global behavior of the system. So previous conclusions, obtained using deterministic models, should remain valid despite the fact that we neglected noise- and space-related issues. This result is not surprising. Intuitively, even if SMB system is heterogeneous (because it is composed of two distinct populations of cells), the spatial distributions of both cells types are uniform; thus, the system evolution is space independent. Nevertheless, we were interested in checking the property using models and simulations. In fact this led us to use and develop original techniques (like Delaunay triangulation or extended to nested membranes systems Gillespie algorithm). From a general point of view, developing such techniques is also of great interest for synthetic biology. Following the concepts of decoupling and abstraction that characterize biosynthetic developments, we have to ideally validate and study designs before constructing them physically. These validations appear at each step of a standard development, at the levels of systems, devices and bricks designing. We tried to follow and contribute to this methodology by considering at first phenomenological and global models, and molecules scaled models at last.

Getting back to SMB, we would like to stress here that these results should be taken with great care, given the extreme simplicity of our models and the lack of data to provide information on parameter values and initial conditions. But still, globally...
**...all these results corroborate our initial design**.

# 6. Paris/Perspectives From IGEM07

SMB is composed of a germline and a soma. Germ cells are responsible for reproduction while the sterile somatic cells, are essential for the germline as the soma exports the DAP compound, supporting germline growth. The special feature of our system, the coexistence of two independent cell types, one dedicated to reproduction and the other sterile, makes it a potentially interesting tool for synthetic biology.

The SMB could also be used to explore basic questions in fundamental biology. If submitted to long time growth, in a chemostat for instance, the SMB could become a model to study how the genome of a cell can evolve leading to changes that do not directly affect the cells phenotype. This system could mimic evolutionary phenomena that are typicall of multicellular organisms.



## 6.1 System improvement through directed evolution

The engineered SMB may be further tuned by laboratory evolution. A key advantage of the system is that selection pressure could be tuned to either force faster reproduction (e.g., improving DAP production and/or export from the soma or alternatively for a more efficient use of DAP within the germline) or for improvement of soma-specific functions (see below). In other words, the soma-germline SMB dichotomy provides us with the possibility to select for functions that are otherwise deleterious for the unicellular organism.

## 6.2 Applications

We envision two immediate applications to the Synthetic Multicellular Bacterium. It could be used as a “metabolic plant” or as “security device”

### 6.2.1 Decoupling reproduction faculty and transgene expression

In the SMB system, any given gene or gene circuit can be selectively expressed in germ cells or somatic cells. Knowing the soma is unreplicative, we are disrupting the connection between the reproduction of the system and the expression of the transgene, leading to potentially interesting applications.

We would like to develop two extensions to the SMB : metabolic engineering and secured genetically modified organisms for environmental release.

## *Security device*

We came up with the idea of modifying the synthetic organism in order to allow, on demand, the full differentiation of the germline into soma in a secured fashion. Why would we want to do that? This possibility is to be considered in the face of biohazard risks. Release of GM organisms in the environment poses ethical as well as technical questions. “Biological security” should be a prime concern in synthetic biology.

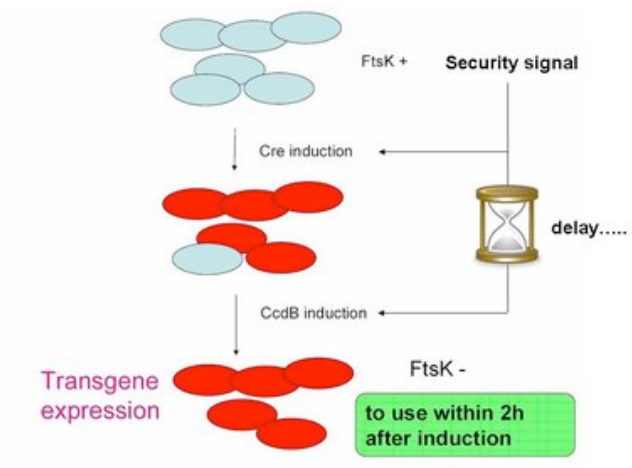
If a task is to be transiently performed by a GM organism in the open environment, a major problem is persistence of the GM organism, with potential risks of proliferation and transgene dissemination. A possible solution, already used for some GM crops, is to render the organisms sterile. Can this solution be implemented for GM bacteria, in bioremediation systems for instance? Using a modified version of our SMB, the answer is yes. The SMB comprises two cell types, one being the soma, a group of cells that have a longer lifetime than wild type E.coli cells & are unable to replicate. The soma cells have the required characteristics for transient use in open environment.

The next consideration is then: can full differentiation be induced in the SMB? In order to achieve this, two events should be initiated:

- Induction of a massive differentiation of the germ line into soma cell
- Followed by selective death of the persistent germ line cells.

Here are details on proposed technical solutions to this challenge:

As indicated earlier, in order for the modified SMB to behave as a secure source of sterile somatic cells, two events should be initiated upon activation of a switch. This could be done in the following manner:




- EVENT 1

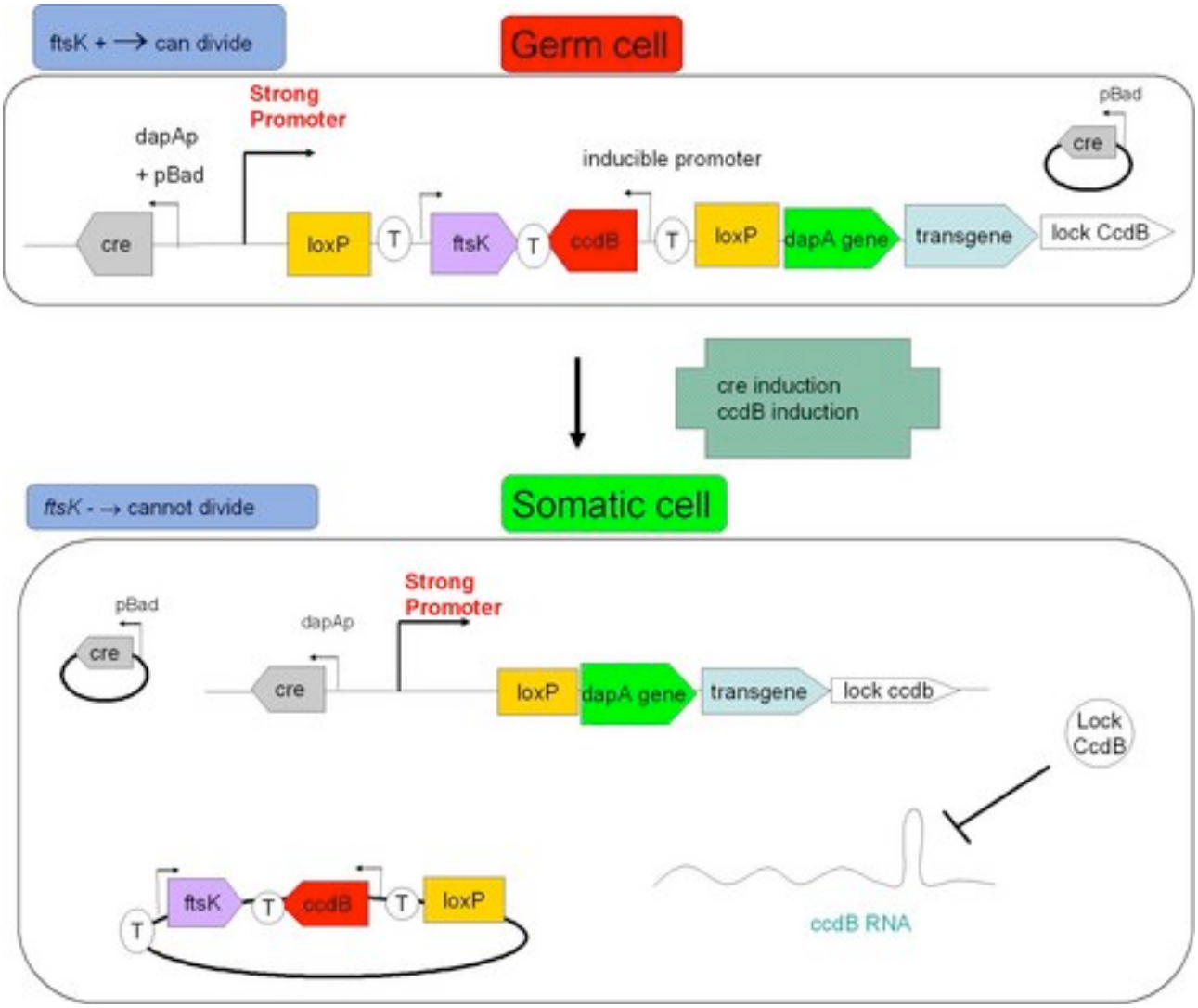
In order to achieve massive induction of recombination, a dormant copy of Cre site specific recombinase could be added to the SMB system. This copy would be plasmid born & would placed under control of an inducible promoter. But a certain proportion of G cells would most certainly persist undifferentiated.

- EVENT 2

The solution to second challenge could be coupling the high recombination switch to a delayed suicide switch in the gem line cells. include the expression of ccdB suicide gene in the germ line cells.

This could be the opportunity to practically apply one of the founding principles of synthetic biology: modularity in systems assembly & organisation. Indeed, a time delay circuit has already been described, constructed & theoretically studied (Sara Hooshangi et al. 2005, Sara Hooshangi et al. 2006 and Juan M. Pedraza et al. 2006). Using such a time delay circuit could allow using a single signal to induce event 1, followed after a delay by event 2.

In addition Size based screening could be performed before environmental release. Indeed, S cells having lost ftsK expression present an interesting phenotype: they grow in size without division taking place, they "filament". The size threshold used in the cell sorting process should be determined by studying size distribution within germ cell and somatic cell populations. This could to improve security.




In the figure above, the recombination procress accompanying differentiation would lead to the transcription of a ccdB lock in somatic cells. This should protect the somatic cells from

the suicide event that germ line cells go through. This lock could be a post transcriptional regulatory mechanism as this class of regulation requires less latency time before appearing.

### *Metabolic engineering*

A bacterial multicellular organism could allow optimizing the production of compounds deleterious to the cell. If one attempts to optimize the production of such a compound in a classical bacteria (E.coli for instance), trade-off appears between the production of your compound and the growth of the cell. The synthetic multicellular organism could in part bypass this problem by partially decoupling growth from synthesis of the exogenous compound. It could indeed be modified so that only the soma produces the noxious molecule. If a mean exists to screen for maximum production of this molecule, you could then select the germline whose soma would have the best exocompound production yield. As long as the production doesn't impair too much the capacity of the soma to feed the germline, the optimization can go on. Thus, a trade-off also exists in this case, but it would very well less stringent that in the wild type E.coli case.

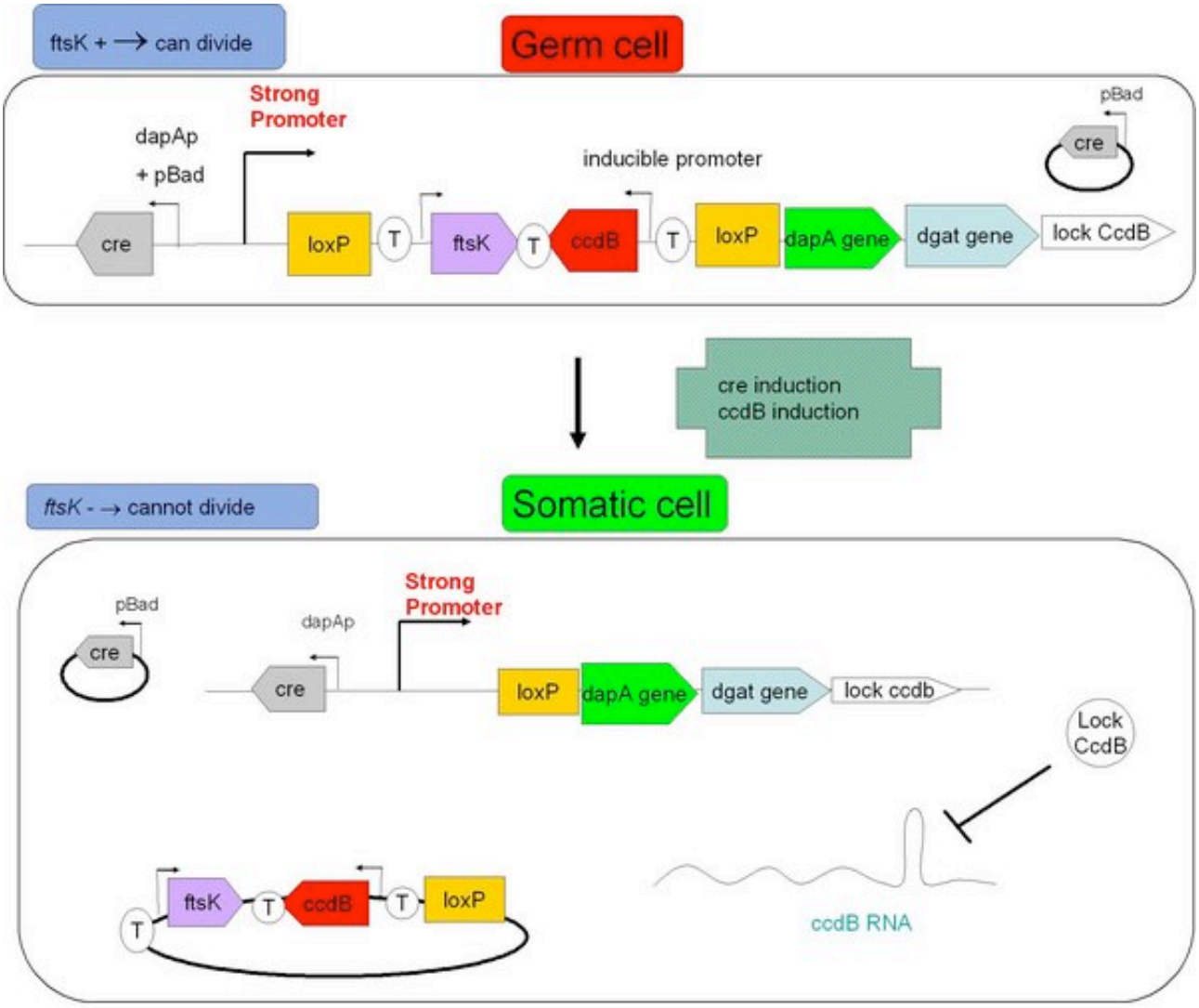
Of course, all this is only possible if the germ line is not affected by the products released by the soma. Compounds that could be optimized in this way are thus constrained to molecules that can be noxious if accumulated in the cell, but that do not affect it too much if in the medium. For instance, if the product can be gradually filtered out of the medium.

## E. Colight: towards a new slim diet

### *Project*

In an attempt to integrate all the proposed applications of the SMB, we advanced towards the construction of Ecolight. Bringing together the synthetic organism (SMB), noxious compound synthesis ("metabolic plant"), and the security device. This would include optimizing the production of triglycerides in the soma cells of our synthetic multicellular bacterium. Soma isolation according to the "security device" would be included. The super triglycerides-producing-not-able-to-divide soma cells could then be ingested. The fatty acids they would store would be as many fatty acids you will not absorb! Eat fat, don't get fat!

E.colight project is also made up with 2 independent parts. First we have to create the SMB to implement the secured module. Second E.coli has to absorb free fatty acid and to synthesize triglycerides in the cytoplasm.

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- BBa_I718002

**Time delay circuit**

- **Ultrasensitivity and noise propagation in a synthetic transcriptional cascade**

Sara Hooshangi, Stephan Thiberge, and Ron Weiss PNAS March 8, 2005 vol.102 no. 10 p3581–3586

- **Noise Propagation in Gene Networks**

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