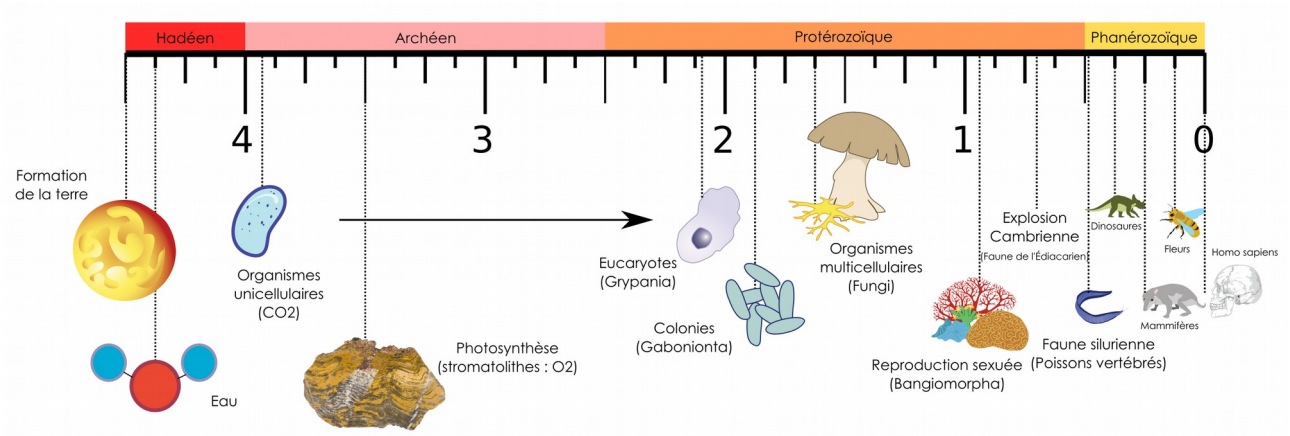
Study of cellular cooperation mechanism : the endosymbiosis

Objective : Recreate « in vitro » the process that led to chloroplasts in plants.

(see abstract in e-mail)

From prokaryotes to eukaryotes: an important transition in the evolutionary history of the living:
Since life emerged 3,8 billion years ago, it has been continuously evolving and colonizing new environments (Huxley, 1942). The first living organisms were **prokaryotes**: unicellular and without a nucleus (Buick, 2008; Mojzsis et al., 1996). This form of life predominated for 2 billion years. Then, two successive important innovations occurred: the **eukaryotes**, unicellular organisms possessing a nucleus (Hedges et al., 2004; Knoll et al., 2006), and the **multicellular organisms** (Bonner, 1998). Our study focuses on the first innovation: the transition from prokaryotes to eukaryotes.



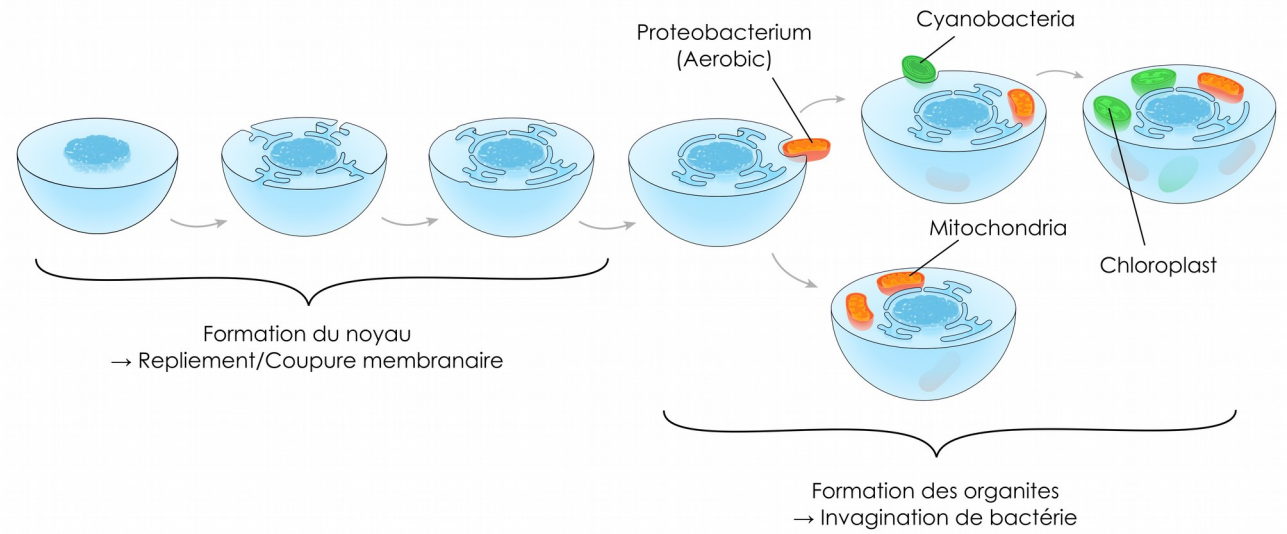
Figures 1 : **Chronology of the evolutionary life history**. Scientific theory representation of events in accordance with the different geo-paleontological observations. The arrow indicates the prokaryotic-eukaryotic transition. Scale in billions of years.

The organelles of the eukaryotes could have originated from an endosymbiosis.
In addition to possessing a nucleus, the eukaryotes, contrarily to prokaryotes, possess specialized structures called organelles, such as mitonchondria and chloroplasts, respectively responsible for cellular respiration (in animals and fungi) and photosynthesis (in plantes and algae). The most consensual hypothesis accounting for the apparition of organelles is the endosymbiosis origin (Martin et al., 2015). This hypothesis postulates that some unicellular prokaryotes, the archea, internalised bacteria with whom they had mutually beneficial relationships. The mitochondria and chloroplasts would have originated from these internalised bacteria. The main elements that support this hypothesis are:

- The organelles possess their own DNA, which is circular, like in bacteria (Timmis et al., 2004).
- They divide independently from the eukaryote cell (Margolin, 2005).
- The organelles DNA has an important degree of similarity with bacterial DNA (Andersson et al., 1998; Dagan et al., 2013).

However, the mechanisms leading to this symbiosis are still unclear. The internalised bacteria could have been initially **preys** which developed a resistance against phagocytosis.

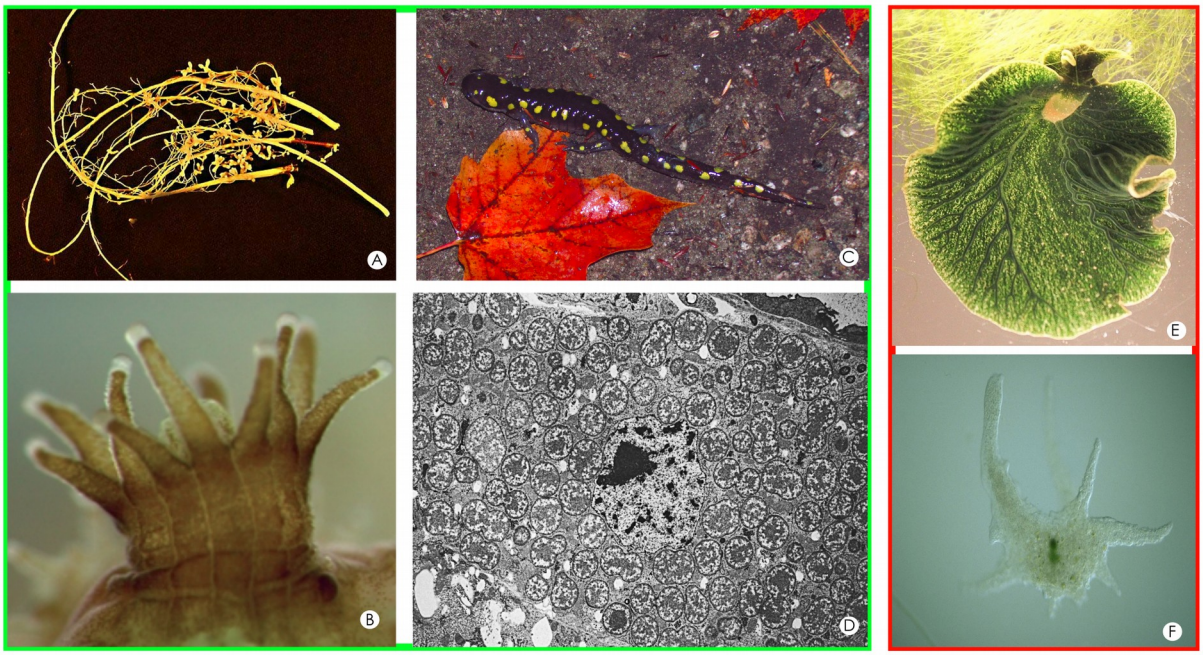
Alternatively, they could have been **parasites** of eukaryote cells before their relationship evolved into a cooperative one. (Zachar et al., 2018).



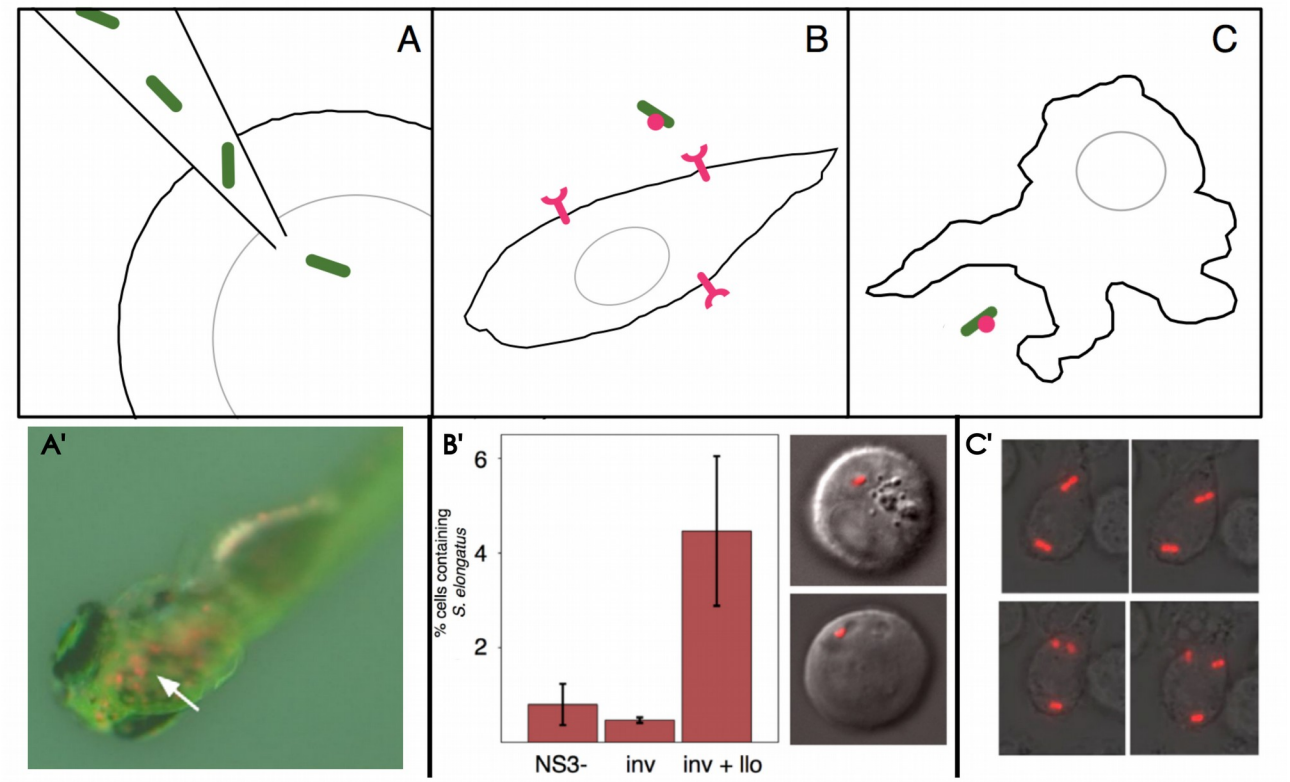
Figures 2: **The endosymbiosis theory.** Above, the path that leads to the Algae and Plants branch and below, the path that leads to the Fungi and the Animals.

Do the instances of endosymbiosis allow us to understand its mechanisms ?:

The observation of the extent endosymbiotic relationships show that the frontier between parasitism and mutuliasm is often blurry. Some relationships are clearly **mutually beneficial**, such as in the relationship between Rhizobia-Fabacees (Markmann and Parniske, 2009), Buchnra-Pucerons (Douglas, 1998), Zooxanthelle-Corail (Venn et al., 2008) et Oophila-Ambystoma (Kerney et al., 2011) (Green box, Figures 3). These relationships started millions years ago and they are necessary to the normal functioning of both involved organisms. Some other relationships are more **ambiguous**: they settle later in the animals development and are optional, as in the case of the sea slime Elysia. This latter feeds upon an algae, and stocks its chroloplasts in its own intestine. Thus this relationship is initially predation of the algae by the slime, although it can also be considered an endosymbiosis between the slime and the algae's chloroplasts (Rumpho et al., 2000). Other cases can be better described as enslavement rather than mutualism, such as in the case of the relationship *Amibea-Candidatus.* When the amoebae are infected by the "candidatus" bacteria, very few survive the epidemia and those who do become dependent to these bacteria (Jeon and Lorch, 1967). These examples show that the endosymbiosis one can observe in the living are complex, diverse, and settled progressively though a long evolutionary history. Thus it is challenging to decipher a posteriori the mechanistic explanation of the settlement of an endosymbiotic relationship. In this respect, building an artificial endosymbiotic relationship can shed light on the mechanisms leading to endosymbiosis.



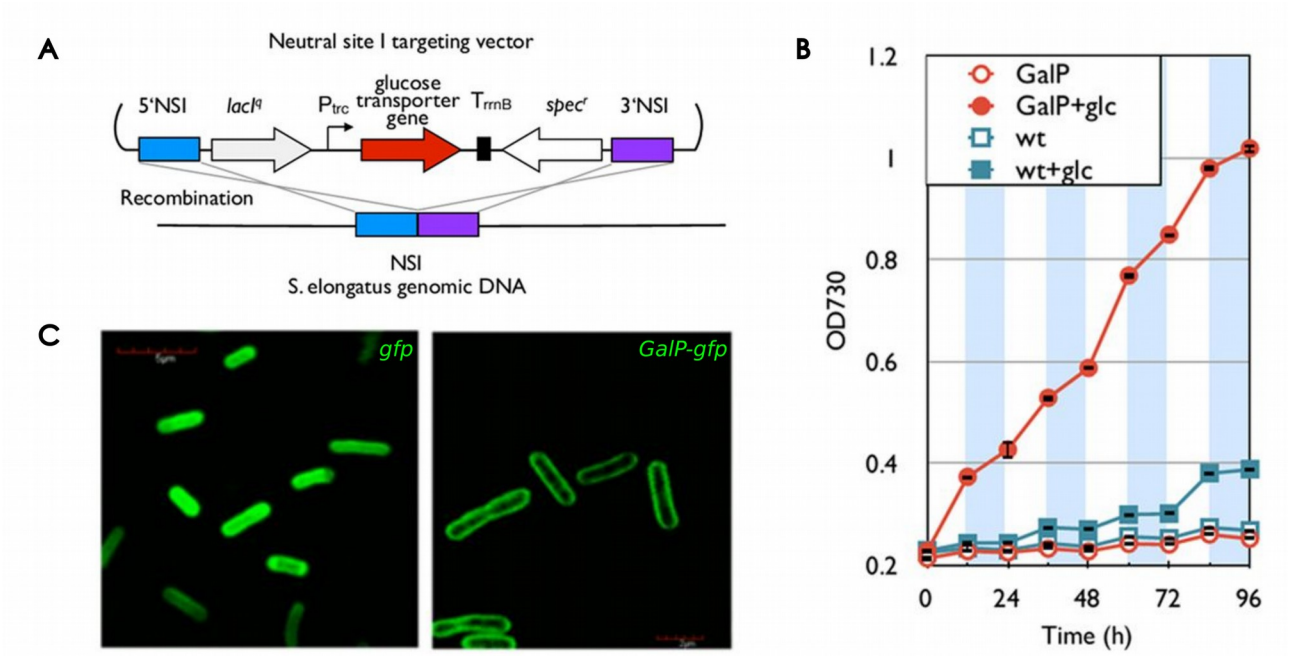
Figures 3: **Diversity of examples of wild endosymbiosis.** In green box, the mutually beneficial interactions with A: Rhizobium on soybean root which allows them to fix nitrogen. B: Zooxanthellae visible in a coral polyp. C: Photograph of spotted salamander in Quebec. D: Buchnera aphidicola cells in a host cell (Hoff, 2007). In a red box, the more ambiguous endosymbiosis interactions with E: Emerald Elysia sea slug consuming the Vaucheria literea alga (Pelletreau et al., 2014). F: Amoeba proteus feeding on phagocytosis. Illustration from Wikipedia.



Figures 4: **Three-way artificial endosymbiosis.** A: Direct microinjection of S. elongatus into zebrafish embryos makes it possible to explore the in vivo dynamics of bacteria inside animal cells. B: Invasion of mammalian cells by the heterologous expression of invasin and listeriolysin O. C: Phagocytosis of bacteria by macrophages. The bacteria then escape from the endosomal compartment by the expression of listeriolysin O. (Agapakis et al., 2011)

Artificial endocytosis as a mean to understand the apparition of chloroplasts

In a study published in 2011, the photosynthetic bacteria Synechococcus élongatus (**PCC 7942**) was considered as a candidate to build an endosymbiosis analogous to the one leading to the existence of the chloroplasts (Agapakis et al., 2011). The PCC7942 are cyanobacteria which were chosen for their experimental convenience (Clerico et al., 2007; Golden et al., 1987) and allowed 3 different endocytosis realisations: the first one by direct **micro-injection** of WT cyanobacteria in a zebrafish egg, and the two others by **co-culturing** cyanobacteria with mammalian cells. In the first case of co-culture, the cyanobacteria were modified to **invade** the mammalian cells (*invasin* of *Yersina Pestis* (Isberg et al., 1987)). In the second cases, they were co-cultivated with macrophages which phagocytose them, but they were modified to **survive** the stay in the phagosomes of macrophages (*listériolysine O* of *Listeria Monocytogène* (Cossart et al., 1989)). These experiments showed that cyanobacteria were able to divide in the cellular cytoplasms (contrarily to most bacteria (Goetz et al., 2001)) without being pathogenic (contrarily to e.coli). However, these studies show that the cyanobacteria die in absence of light. In this respect, it cannot be considered as a complete endosymbiosis relationship because the host-bacteria interactions are limited : the bacteria survive by its own metabolism using photosynthesis. Neither the bacteria nor the host draw significant benefits from the co-habitation. Is it possible to make the ***PCC-7942 able to benefit significantly from the nutrients provided by the host cell in a way it can survive in absence of light?***



Figures 5: **Glucose transporter insertion to S. elongatus.** A: Schematic representation of the integration of the glucose transporter gene into the genome of S. elongatus. B: Growth curve of the galP (red) and wild type (blue) strain with and without 5 g / liter of glucose. C: Confocal microscope images of the gfp strain (left) and the galP-gfp strain (right). (McEwen et al., 2013)

Modified cyanobacteria able to survive in the dark

The *Synechococcus élongatus* cyanobacteria are able to produce organic matter from light and CO2 but they are not able to consume carbon compounds (**strictly photo-autotrophic**) (Chen and Chen, 2006). There are two hypotheses to explain this inability: either the cell **membranes** are impermeable to sugars (Zaslavskaia et al., 2001), either the sugar **synthesis cycle** is incomplete in the cell (Zhang et al., 1998). In a study published in 2013, the authors tested the first hypothesis by creating several mutant lines which produce glucose transporters (McEwen et al., 2013). Their results show that the addition of the glucose transporter **GalP** (E.Coli (Henderson et al., 1977)) made the cells **heterotrophic**. These cells are thus able to store the excedent glucose in glycogen form, which increases three-fold their speed of growth. **This cell being able to metabolize glucose, would it be able to benefit from the endocytosis by a**

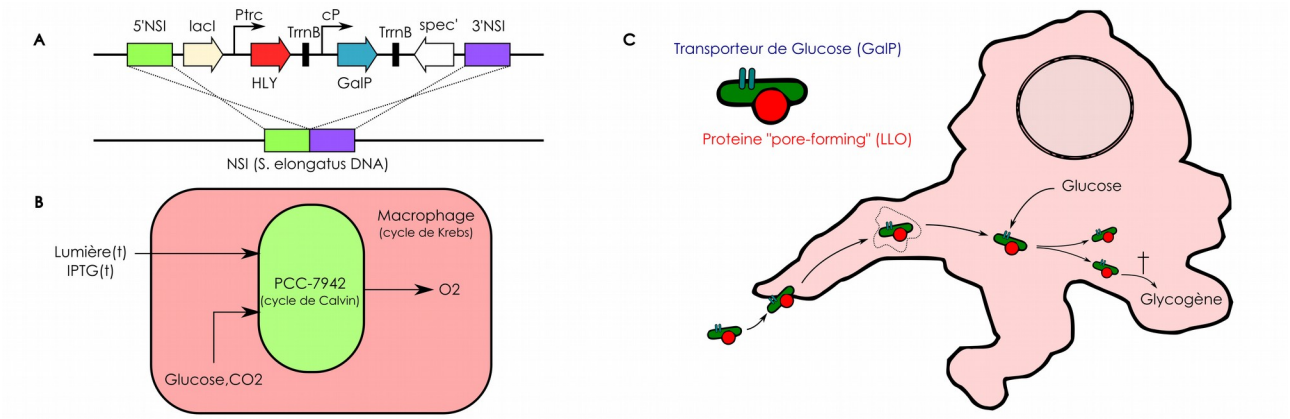
mammalian cell? Indeed, in the dark, the bacteria could use the glucose produced by the mammalian cell.

Objective :

Two approaches can be considered to build a stable artificial endosymbiosis. The first one consist in starting from a wild type situation where the host cell **predates** on the bacteria and provide the bacteria with a survival mechanism. The second consists in starting from a situation where it is the bacteria that kills the host (**parasitism**) and provides the host with a survival mechanism. For this project I will focus on the first approach. Using the two previous complementary studies, I propose to

1) Modify the cyanobacteria PCC7942 in a way that allows it to survive in the host cell by providing it a defense mechanism against phagocytosis. In addition, the cyanobacteria will also be provided with a glucose transporter in order to take advantage from the nutrient present in the host cytoplasm in the absence of light
2) Co-cultivate the modified cyanobacteria with macrophages.

Thus we would obtain a model that allows to explore different aspects of the hypothesis of chloroplasts originating from endosymbiosis. However, the line between "endosymbiotic relationship" and "organelles" is still not well defined (Gruber). How will this relationship evolve? Will the macrophage draw some benefit from this endosymbiosis? Will the relationship end up being interdependent?



Figures 6: **Survival model of cyanobacteria in mammalian macrophages.** A: Schematic representation of the integration of a possible regulatory circuit for survival in the cytoplasm. B: Systemic representation of the interactions between cyanobacteria and host cell. C: Representation of the endocytosis of mutant cyanobacteria.

Experimental approach:

1. Make a strain of cyanobacteria able to metabolize the glucose:

I have built a strain able to metabolise the glucose, based on the strain described in McEwen article. My observations were in agreement with the previous results that this strain grows three times faster in presence of glucose, and can survive in absence of light in a culture medium BG-11 (Stanier et al., 1971). I used the vector pAM2991 (Ivleva et al., 2005) for the homologous recombination in which I introduced the GalP sequence, expressed constitutively unlike the strain of the previous article.

2. Addition of a survival mechanism in the phagosomes:

The incorporation of the listeriolysine O sequence allows to create pores in the phagosomes, which frees the cyanobacteria in the cytoplasm. This sequence should not be expressed constitutively or it would alter the defence mechanisms of the macrophages on the long run (Hamon et al., 2012). Several approaches can be considered to control its production and to restrict it to the entrance of cyanobacteria in the macrophages: the first method is to activate the production of the permease only when IPTG is present in the medium (Ptrc/LacI). The second method would to be add a Lox sequence to the vector and to remove the permease sequence after the it is released in the cytoplasm. Last method, more complex, would be to regulate the expression of the permease with an acidity marker (the phagosome is acidic).

Co-culture of mutant cyanobacteria with immortalized macrophage strains (RAW 264.7) in DMEM-FBS medium

The absence of pathogenic effect will be tested by comparing the growth of RAW 264.7 (Raschke et al., 1978) macrophages with and without endocytosis of cyanobacteria. The stability of this endosymbiosis will be monitored on the long run.

Nom	Objets	Description
E.Coli	Bacteria	Contains glucose transporter sequence
PCC-7942	Bacteria	Photoautotrophic strains to modify (Franck Chauvat)
RAW 264.7	Mouse cells	Macrophage; Abelson murine leukemia virus transformed (ATCC – lgcstandards)
pAM2991	Plasmides	Cyanobacteria cloning vector (Susan Golden, Addgene)
PAD-hly-Myc	Plasmides	Vector with LLO coding sequence (Alice Lebreton)
GalP	Proteins	3'-AACACCTGCCTCCAATTGATGCCTGACGCTAAAAAA 5'-TTCACCTGCCTCTTCCTAGTATTAATCGTGAGCGCCTAT
hly/llO	Proteins	3'-AGCACCTGCCTCGAGGAGGAAAAACATATGAAAAAAATAATGCTA 5-CCCACCTGCCTCAGATCTTTATTCGATTGGATTATCTACTTTATTACTATATTTCGG
BG-11	Medium	100x concentrate, sterile filtered (Merck – sigmaaldrich)
DMEM	Medium	Low glucose, pyruvate, no glutamine, no phenol red (Thermofisher)

The possible applications:

In addition to contributing to address fundamental scientific questions, the construction of a stable endosymbiotic strain could also have applications in the medical domain. The invasive bacteria have already been considered as candidate biological tools to destroy tumours (Anderson et al., 2006; Forbes, 2010), for the transfer of functional genes (Grillot-Courvalin et al., 1998), gene therapy (Xiang et al., 2006), or for the delivery of peptides/nucleotides for vaccines (Bermudes et al., 2002). The e.coli bacteria were until now the unique candidate explored in these studies. Yet, e.coli is a bacteria with fast proliferation, and thus has more chance to alter the normal functioning of the host organism than a slower proliferation bacteria such as the PCC7942 cyanobacteria. A cyanobacteria in symbiosis with human cells could present therapeutic interest. For example, in the case of tumours, one could consider providing the cyanobacteria with a host cell destruction function (Perforine (Law et al., 2010)) which would be regulated by the cellular oxygen level (hypoxia promotor fdhF (Wang and Gunsalus, 2003)).

Conclusion :

This project would enable to create photosynthetic bacteria able to invade the cytoplasms of mammalian cells. The obtention of a stable strain would contribute to the understanding of the mechanisms which endosymbiosis stem from. Also, it will allow us to control the levels of host / symbiont integration, which could allow us to know what distinguishes endosymbiotic relationships from organelles. The first steps of molecular biology design were already realised in parallel of my thesis project. The remaining steps are the addition of a survival mechanism in host cells for the cyanobacteria and the co-culture with immortalized mammalian cells. The energetic needs of an immortalized mammalian cell correspond to the glucose production of 25 to 14 000 cyanobacteria (Ducat et al., 2011; Niederholtmeyer et al., 2010). Thus, it is left to quantify the benefit the mammalian cells can really draw from this endosymbiosis, if any. What we could achieve here in two years, nature took 2 billion years to see the first organelles of eukaryotes appear, according to our model and by comparison, would there be today traces of the origin of endosymbiosis? Last, if these artificial bacteria prove to be non-pathogenic or immunogenic, they can present a great potential as tools for medical applications.